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PRINCIPAL INVESTIGATOR: I. A. Bernstein, Ph.D.

CONTRACTING ORGANIZATION:
1528 Public Health I
The University of Michigan
109 S. Observatory
Ann Arbor, MI
48109-2029

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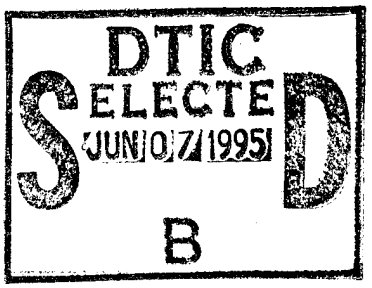

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I. A. Bernstein, Ph.D.

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The University of Michigan
1528 Public Health I
109 S. Observatory
Ann Arbor, MI 48109-2029

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13. ABSTRACT *(Maximum 200 words)*

The mission of this project was to determine the cellular and molecular lesions associated with cutaneous vesication from bis(2-chloroethyl)sulfide (BCES). Cultures of keratinocytes were used to focus attention on the direct interactions between the mustard and its epidermal targets. The technical objectives included confirming that DNA was the primary molecular target of BCES in human epidermal keratinocytes, identifying and quantifying BCES-mediated DNA-adducts in relation to dose, determining why epidermal basal cells are more susceptibl to BCES than differentiated cells, and investigating the possible role of informational error in DNA in the cytopathogenic process. Data generated in the project suggest that (a) DNA is the primary epidermal target of BCES, and the fidelity of DNA repair governs survival of the germinative population, and (b) BCES causes a decrease in the germinative population by cellular differentiation, as indicated by the appearance of mature keratin protein, as well as necrosis.

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I. FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

In conducting research using animals, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigator adhered to policies of applicable Federal Law 45 CFR 46.

I. A. Bernstein, Ph.D. October 31, 1994

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

II. SUMMARY

This project seeks to determine the cellular and molecular lesions that are associated with blistering in the epidermis when human skin is exposed topically to bis(2-chloroethyl)sulfide (BCES). The study is being done using cultures of cutaneous keratinocytes in order to focus on the direct interactions between the mustard and its cellular targets. The technical objectives of the project are (a) to confirm in human cell cultures the primacy of DNA damage, repair, and informational error in the initiation of cutaneous pathogenicity; (b) to identify and quantify the BCES-mediated adducts in the DNA of keratinocytes exposed to the mustard in relation to the level of exposure; (c) to determine why basal cells are more susceptible to damage from BCES than are differentiated cells, and (d) to establish the nature of the informational lesion in DNA which is a factor in the progression of the epidermal pathogenic response. An understanding of the events that initiate pathogenicity might identify therapeutic measures which could be taken to minimize or obviate the response.

A summary of results obtained in this investigation includes the following: (a) the half-life of BCES is 21-22 min at 37°C in a phosphate buffered saline solution and only about 8 min if a submerged, stratified culture of keratinocytes is included; (b) the biosynthesis of DNA is inhibited more drastically than is RNA or protein synthesis in a lifted culture of human keratinocytes (i.e., pseudo-epidermis) exposed to a low-level of BCES; (c) in submerged cultures of human keratinocytes and in isolated DNA from human keratinocytes, exposure to between 300 and 5000 uM BCES, results in the appearance of the N^7-mono-adduct of guanine as the major alkylation product and smaller amounts of the di-guanyl-adduct as well as other adducts; (d) exposure of submerged cultures of rat keratinocytes exposed to low-levels of BCES, provides no support for the hypothesis that a dramatic decrease in the level of NAD (NAD^+ + NADH) is responsible for the cytotoxicity; (e) the level of interstrand cross-linking in the DNA of basal and differentiated cells is similar and no evidence has been found to suggest that repair of such cross-links is better in human differentiated calls as compared with basal cells; (f) the level of interstrand cross-linking in human keratinocytes in submerged culture immediately after exposure correlates positively with the level of interleuken-1α in the residual cells or the medium 72 hr later as well as with the percentage of cells in the culture that do not exclude trypan blue, and (g) exposure of monolayer cultures of rat keratinocytes results in the precocious differentiation of germinative cells as evidenced by the appearance of keratin that is characteristic of differentiated - but not basal - cells in the intact cutaneous epidermis.

III. NARRATIVE REPORT OF PROJECT PROGRESS

A. Introduction: Status of the Field at the Beginning of the Project

1. Effect of topical exposure to BCES in the human in vivo

Application of bis(2-chloroethyl)sulfide (BCES) to human skin results in an initial erythema followed by blistering. In the formation of a blister, fluid accumulation is said (Stoughton, 1971) to be secondary to fundamental damage to the cellular structures and to completely replace the pre-existing tissue structure. The blister is capped by a part or all of the epidermis. BCES-induced vesication initially involves separation at the dermal-epidermal junction and destruction of the basal and lower spinous layers of the epidermis (Warthin and Weller, 1919; Sinclair, 1949). Later in the destructive process, the necrosis may spread to the upper spinous and granular layers as well as into the dermis (Sulzberger, et al., 1947; Warthin and Weller, 1919; Sinclair, 1949). From the time course of dermatopathologic development and the time post-exposure within which the process can be reversed, it is clear that the molecular course of the pathologic process in vivo, is set within the first 3 min of exposure. The pathogenesis of the skin lesion in vivo is complex (Vogt, et al., 1984) being influenced by systemic factors and by the inflammatory process, thus making it difficult to determine the early molecular events and the nature of the events in the target tissue which actually lead to the death of cells in the basal layer. This project is using cell cultures in its efforts to obviate these confounding influences and to define the nature of the molecular and cellular sequelae that follow exposure to BCES.

2. Cutaneous toxicology of BCES in vitro

a. Biochemical effects

Exposure of isolated skin to a high-level of a vesicant for as little as 5 min, can result in the inhibition of glycolysis and respiration (Barron, et al., 1948). Glycolysis is inhibited by a lower exposure than is respiration. In the case of exposure to mustards, there is a concomitant reduced level of pyridine nucleotides in the cell (Holzer and Kroger, 1958; Frazer, 1960). Recent evidence suggests that this effect occurs by virtue of the stimulation of polyADP polymerase and consequent loss of NAD^+ (Gross, et al., 1983, 1985; Papirmeister, et al., 1991; Smith, et al., 1990) although not all studies support this hypothesis (C.F., Mol, et al., 1991; Martens, 1991).

b. Development of the "lifted" culture epidermal model (i.e., pseudo-epidermis)

Methods have been developed in this laboratory for routinely obtaining primary human (Bernstam, et al., 1990) and animal (Vaughan, et al., 1986; Bernstam, et al., 1986) stratified, cornified cutaneous cultures, which mimic the structure and have most of the biochemical characteristics of the epidermis, in situ. Ordered stratification and extensive cornification is obtained by "lifting" the culture to the air-liquid interface. The appropriateness

and credibility of this system, for the investigation of the initial phases of mustard toxicity, were established when it was demonstrated that application of a low concentration of BCES to the surface of the pseudo-epidermis resulted in necrosis of the basal cells by 2 days post-exposure while the differentiated cell layers seemingly remained unaffected (Bernstein, et al., 1987; Scavarelli-Karantsavelos, 1989), as occurs in the cutaneous epidermis after exposure to BCES in vivo.

c. Epidermal DNA as the primary target of BCES toxicity

The molecular target in the culture that appears to be most susceptible to damage from BCES is DNA (Vaughan, et al., 1988) as it appears to be in vivo (cf., Fox and Scott, 1980). Vaughan, et al. (1988) showed that inhibition of [^{3}H]thymidine ([^{3}H]TdR) incorporation into the DNA of cells in the "lifted" cultures of rat keratinocytes occurred after the topical application of a lower concentration of BCES (0.01 nmol/cm^2) than was necessary (10 nmol/cm^2) to inhibit the incorporation of [^{3}H]uridine ([^{3}H]UR) into RNA or [^{14}C]leucine ([^{14}C]leu) into protein. At an exposure of 10 nmol/cm, semi-conservative replication of DNA was completely inhibited (Zaman-Saroya, 1989). In submerged monolayer cultures of rat keratinocytes, an exposure of 20 uM BCES did not inhibit the incorporation of [^{14}C]leu but inhibited the incorporation of [^{3}H]UR by 15% and [^{3}H]TdR by 80% (Ribeiro, 1988).

d. Initiation of BCES pathogenicity studied in vitro

The pathogenicity of mustard in molecular terms is still unclear but this substance is a powerful alkylating agent of DNA, RNA and protein. BCES alkylates and cross-links the purine bases in DNA. Alkylation of the phosphate groups may also occur. It has been reported (Brookes and Lawley, 1961; Papirmeister, 1961; cf., Papirmeister, et al., 1985) that in neutral solution about 60% of alkylation of DNA by BCES involves monofunctional addition at guanine N^7, 15% involves intra- or interstrand bifunctional addition between the N^7 position of two nearby guanine residues. and 15% involves monofunctional addition at N^3 of adenine. Alkylation at the O^6 position of guanine has been reported by Ludlum, et al. (1984). Minor alkylation at other sites also occurs.

The literature reports quantitative studies on the adducts in the DNA from exposed Ehrlich ascites tumor cells, salmon sperm, yeast, bacteriophage and human white blood cells as well as on the adducts found in the DNA isolated from calf thymus, bacteriophage and salmon sperm and then exposed. The ratio of di-adduct to mono-adduct of guanine has been reported to be about 0.2.

1) Alkylation and repair of DNA

Extensive alkylation of DNA could lead to generalized breakdown of the badly damaged DNA resulting in cell death (cf Wheeler, 1962). A lesser degree of alkylation might allow repair of the damaged DNA but with low fidelity so that mutations could be inserted resulting in disruption of normal metabolic function, inhibition of DNA synthesis and delay or cessation of mitosis (cf Wheeler, 1962). Proteins can also be

alkylated (Ross, 1962) but damaged proteins can be replaced whereas damaged DNA may be irrevocably harmed suggesting that alkylation of DNA is more likely to initiate pathogenesis from BCES than alkylation of protein. Similarly, alkylation of RNA is not likely to be responsible for initiating the toxic response. Papirmeister, et al. (1985) have proposed a molecular model for the pathogenesis of BCES-mediated cutaneous injury that starts with the alkylation of DNA and is followed by the insertion of single-strand breaks in the nucleic acid; activation of a poly ADP-ribosylation mechanism that leads to depletion of NAD^+ (Meier, et al.,1987); metabolic disruption; release of proteases, and finally generalized cellular necrosis.

In bacteria, the ability to excise BCES-alkylated products from DNA is associated with increased resistance to the mustard (Lawley and Brookes, 1968). Roberts, et al. (1971) concluded that both mono- and bifunctional adducts can be removed from DNA. However, there appears to be disagreement as to the relative rates at which repair of the two types of lesions occurs (Reid and Walker, 1969; Roberts, et al., 1971). It seems likely that repair of bifunctional adducts would occur at a slower rate and with less fidelity than would repair of monofunctional adducts.

2) Structural and metabolic effects on DNA

Recent investigation in this laboratory has utilized the submerged monolayer culture, grown in a "low- calcium" medium (cf., Hennings, et al., 1980), to study the effects of BCES on the structure and metabolism of DNA during the first 6 days post-exposure. The normal kinetics of DNA replication and mitosis in this culture have been defined for that period (Ku and Bernstein, 1988a).

Submerged monolayered cultures exposed to 1 uM BCES at 24 hours after seeding, showed a progressive inhibition of TdR incorporation into the DNA with time post-exposure. Cells in S phase at the time of exposure to BCES incorporated [^{3}H]TdR as they slowly completed DNA replication (Ribeiro, 1988). However, no cells entered the S phase of the cell cycle from the time of exposure until sometime between 48 and 72 hours later (Ku and Bernstein, 1988b). Single-strand breakage (SSB) in DNA was observed by the nucleoid sedimentation technique (cf., Cook, et al, 1976; Romagna, et al., 1985) immediately after exposure of the culture to as little as 0.1 uM BCES. However, when exposed cultures were washed and allowed to incubate in fresh medium, the nucleoid sedimentation value became normal by 22 hr after exposure to as much as 5 uM BCES (Ribeiro, Mitra and Bernstein, 1991). If this return of the nucleoid sedimentation value to the normal level represented the restoration of the gross structure of DNA and not, as might be the case, the insertion of DNA-DNA or DNA-protein crosslinks, this repair process was insufficient to allow the cultures to resume their normal course of DNA replication and mitosis. Either BCES at this level of exposure inhibits the enzymatic machinery for DNA replication and cell division directly or the genomic information required for these processes is still aberrant and several days are required to restore the integrity of DNA in this regard.

Similarly, when lifted cultures were exposed topically to BCES, SSB were found in the DNA of both the germinative and differentiated cells immediately post-

exposure (Scavarelli-Karantsavelos, 1989). It took a dose of 50 nmol/cm^2 applied to the surface of the culture to obtain even a minimal number of SSB in the differentiated cell population while 10 nmol/cm^2 was sufficient to get the same effect in the basal cells. Since SSB in DNA result from DNA repair as well as non-enzymatic hydrolysis, these data suggest that alkylation of DNA occurs to a greater degree in basal than in differentiated cells. Supporting this conclusion was the observation that the basal cell population showed a higher degree of radioactivity in the DNA than did the differentiated cells immediatly after [^{14}C]BCES was topically applied to the lifted culture (Scavarelli-Karantsavelos, 1989).

It was initially assumed that the incorporation of [^{3}H]TdR could be used to monitor the repair of DNA damaged by BCES. However, when cells in monolayer cultures, synchronized and being held at the G1-S boundary of the cell cycle by the presence of aphidicolon (Aph) (Longiaru, et al., 1979; Spadari, et al., 1984), were exposed to 1, 5 or 20 uM BCES and then allowed to enter S phase by removal of the Aph, [^{3}H]TdR was not incorporated into the parental DNA in any measurable quantity. In these experiments (Ribeiro, 1988), bromodeoxyuridine was added with the tritiated precursor so that the parental DNA, which should have become labeled if excision repair had occurred, could be separated from replicated DNA by CsCl density gradient centrifugation. The small amount of [^{3}H]TdR that was incorporated was exclusively in the replicated DNA. However, when [^{3}H]deoxy-guanosine ([^{3}H]GdR) was substituted for the labeled TdR, considerable incorporation of tracer occurred in the parental DNA. [^{3}H]GdR also labeled the replicated DNA to some extent. This preliminary information makes it appear that a mechanism of repair other than the classical excision repair is responsible for correcting the damage in DNA resulting from exposure to BCES. This latter mechanism could involve the direct replacement of damaged guanine residues which are the major sites of adduct formation with the BCES (cf., above). Such a mechanism was reported by Hennings, et al. (1974) and Hennings and Michael (1976) in mouse skin cells exposed in vitro to beta-propiolactone and N-methyl-N'-nitro-N-nitrosoguanidine. In the latter case, at low exposure, only GdR was incorporated into DNA; at more toxic doses, both GdR and TdR were incorporated. This suggests that the cell limits its repair to the purine base if the damage is minimal as probably occurs at the low levels of exposure to BCES used in experiments done in this laboratory. At greater exposure which produces greater damage, long stretches of nucleotides are probably excised and repaired by the better known excision repair system. The mechanism and relevance to pathogenicity of these mechanisms of repair need further study.

3) Morphological effects

Although an effect on the integrity of DNA was seen in submerged monolayered cultures by 1 hour after exposure to 0.1 uM BCES, morphological changes were seen only much later or after exposure to higher levels of the mustard. The appearance of unusually large cells in treated cultures has been observed to be a morphological indicator of exposure to BCES (Brewer, et al., 1961; Crathorn and Roberts, 1965, 1966; Roberts, et al., 1971; Ku and Bernstein, 1988b). These large cells, which were detectable in monolayered cultures 48 hours post-exposure to 1 uM BCES, were the

predominant cell type 4 days later (Ku, 1987). As the level of exposure was increased, these cells became the major type earlier. They appeared to be differentiated since they carried glycoconjugates on their outer membranes which seemed to be identical with those of spinous cells and they did not replicate their DNA (Ku and Bernstein, 1988b). At 48 hours post-exposure in cultures exposed to 20 uM BCES, when the culture contained 33% or fewer cells as compared with unexposed cultures and most of the cells were the large type, the cells were seen to contain an unusually large number of small mitochondria, some mitochondria showing degeneration (Brown and Bernstein, unpublished). The treated cells also had a large number of vacuoles. In spite of this evidence for mitochondrial damage, previous work of this project (cf., Bernstein, et al., 1985) had demonstrated that an abnormality in mitochondrial respiration required exposure to 250 uM BCES.

4) Metabolic abnormality

Exposure to BCES does result in some type of abnormality that interferes with the normal balance of proliferation and differentiation in the epidermis (Ku and Bernstein, 1988b). The finding of an abnormal protein - probably a keratin - in what appear to be differentiated cells of monolayered cultures at 24 hours post-exposure to 10 uM BCES (Locey and Bernstein, 1987) supports the view that sulfur mustard can affect differentiation as well as proliferation.

A monoclonal antibody (2D6), obtained in this laboratory, decorates only the basal cells in normal epidermis or in unexposed "lifted" keratinocyte cultures (Bernstam, et al., 1990) from the rat. However, this antibody binds to a 55 kD keratin which has been found in both basal and differentiated cells. During the first day of growth of submerged monolayer cultures, the percentage of cells that are decorated with 2D6 goes from 95% at seeding to about 60% at 24 hours as the culture shows partial differentiation (Ku and Bernstein, 1988a). Presumably, 2D6 is not binding to differentiated cells.

In cultures exposed to 10 uM BCES and incubated for 24 hours, there was a loss of about 33% of the cell number but many of the remaining cells were larger than normal and bound 2D6. Polyacrylamide gel electrophoresis and immunoblotting showed that the antigen for 2D6 in these exposed cells had a kD value of 59.

3. Summary of basis for hypothesis

It appears from studies of the effects of BCES in cultures of rat keratinocytes that alkylation of DNA initiates the pathogenic process. Basal cells are more affected than differentiated cells. Although restoration of the gross integrity of DNA appears to be accomplished at low levels of BCES, exposed cultures do not resume normal proliferation and differentiation. Abnormal large differentiated cells become predominant as normal cells are lost. An abnormal protein - probably a keratin - appears in these cells. These effects appear earlier as the level of BCES is increased. The possibility that these effects represent the insertion of informational error in the DNA of cells exposed to BCES is supported by the report by Fan and Bernstein (1991) which states that African green monkey kidney (AGMK)

cells exposed to relatively low levels of BCES exhibit a dose-responsive decrement in their ability to repair mismatched base pairs in DNA.

4 Hypothesis for this project

The following hypothesis was tested in this project:

The pathogenic response to BCES in the pseudo-epidermis, as in the skin topically exposed to the mustard, is initiated by alkylation of DNA in the epidermal germinative (basal) cells and consumated by the necrosis of this population of cells. However, the severity of the overall toxic reaction depends primarily on the nature and degree of injury to DNA and the cell's ability to repair the structural and informational damage originally inflicted on the DNA by the alkylation - parameters which are affected by the level of mustard to which the cell is exposed.

B. Methods

1. Assay for residual BCES

The assay used to determine residual BCES involved extraction of the remaining mustard from the aqueous phase three times with an equal volume of $CHCl_3$. Determination of the mustard in a 20 ul-aliquot of the organic phase was done by reaction for 5 min at 95°C with 0.4% 4-(p-nitrobenzyl)pyridine in a mixture of 5.6% sodium perchlorate monohydrate and 0.05% acetic acid in 2-methoxyethanol and determination of the absorbance generated at 560nm by subsequent incubation for 30 sec with 0.05 vol of piperidine. Absorbance was converted to concentration by reference to a standard curve, i.e., an A_{560nm} of 0.01 approximated 210 ng of BCES per 20 ul of CHCl-extract.

2. Growth, exposure to BCES and labeling of stratified, cornified cultures

Lifted stratified, cornified cultures on nylon microporous membranes, grown as described by Bernstam, et al. (1990), were exposed to mustard by topical application of 50 ul of BCES in 70% dimethylsulfoxide (DMSO) or in phosphate buffered saline (PBS) for 30 minutes at 37°C. The cultures were then washed three times in Eagle`s Balanced Salt Solution (EBSS) or PBS and were labeled as noted below or were further incubated in growth medium at the air-liquid interface for 48 hr at 37°C. At the end of the incubation period, cultures were exposed at 37°C for 4 hr to the labeled precursor (i.e., [^{3}H]TdR, [^{3}H]UR or [^{14}C]leu) by adding the tracer to the medium (MEM) and positioning the membrane at the interface between the MEM and the atmosphere. Cultures were then washed with EBSS or PBS and treated with 10% trichloroacetic acid. The resulting precipitate was dissolved in NaOH and counted for ^{3}H or ^{14}C as described by Vaughan, et al., (1988).

3. Growth of monolayer cultures and obtaining specifically labeled DNA

The protocol used for labeling and isolating the cellular DNA was as follows:

Monolayer cultures of keratinocytes, derived from rat skin as described by Ku and Bernstein (1988), were grown submerged in normal levels of calcium for 24 hours and then were exposed to [^{3}H]TdR, [^{3}H]GdR, [^{3}H]deoxycytidine ([^{3}H]CdR) or [^{3}H]deoxyadenosine ([^{3}H]AdR) for 24-36 hr. The medium was aspirated from each batch of culture dishes and the cultures were washed three times with PBS. After the last wash was completed and the PBS was aspirated, the cells were detached from the dish using a rubber policeman, were suspended in PBS and were recovered by sedimentation at 900xg for 15 min at 2-4°C.

It was not possible to obtain [^{3}H]GdR commercially. Therefore, [^{3}H]GdR triphospate (Sigma) was purchased and enzymatically hydrolyzed to the deoxynucleoside. The labeled GdR was isolated by high performance liquid chromatography (HPLC). The procedure used to isolate and purify DNA from the cultured cells was as described in detail by Davis, et al. (1986). In experiments done in the latter half of the contract period, [^{3}H]guanosine

([^{3}H]GR), rather than [^{3}H]GdR, was used to label the guanine moieties of DNA. Both tracers were shown to produce only labeled guanine in the isolated DNA.

The cellular pellet was suspended in 10 vol of a proteolytic solution containing 0.01 M Tris buffer, pH 7.4, 0.01 M EDTA and 1 mg of Proteinase K (Sigma) per ml and was incubated for 20 min at 65°C followed by slow shaking at 37°C for 18-24 hr. During this process the cells disintegrated and the DNA was liberated into the solution.

The solution was extracted with salt-saturated phenol containing Tris buffer, pH 7.4, m-cresol, EDTA and 8-hydroxyquinoline to separate the nucleic acids from protein. The protein precipitated and accumulated at the interface between the organic and aqueous phase. Sodium acetate, pH 7.4, was added to the aqueous phase and the DNA was precipited in 70% ethanol. The precipitate was sedimented and any RNA present was destroyed by incubation with RNase (DNase-free). The purified DNA was recovered by precipitation in 70% ethanol as before.

To ascertain that the ^{3}H indeed represented incorporation of the labeled base into the DNA (rather than contaminating precursor-nucleoside), an aliquot of the labeled DNA was mixed with a much larger amount of unlabeled DNA, the total DNA was successively precipitated and redissolved a number of times, and a sample of the DNA in each precipitate was hydrolyzed and analyzed for the ^{3}H in the labeled base utilizing the HPLC procedures described below and liquid scintillation counting. In the case of DNA labeled in the thymine moiety, the specific activities (cpm/nmol of thymine) found for the precipitates were 34.9, 37.7, 36.1 and 35.3 in precipitates 1, 2, 3 and 4, respectively. Clearly, the labeled base is incorporated into the DNA by the procedures used and is not present as a contaminant.

DNA labeled with [^{3}H]TdR, [^{3}H]GdR or [^{3}H]guanosine ([^{3}H]GR) or [^{3}H]CdR was found to be labeled only in the base corresponding to the precursor used. In the case of [^{3}H]AdR, both the adenine and guanine moieties of the DNA were labeled.

4. HPLC procedure

HPLC was carried out on a Varian Model 5000 HPLC apparatus (Varian, Palo Alto, CA) using the following system: Elution system - 31 mM KH_2PO_4, pH 7.0 and absolute methanol (HPLC grade); Column - reversed phase, C_{18} ODS 2, Spherisorb (Phase Separations, Norwalk CT); Temperature - ambient; Flow rate - 1 ml/min; Program - sample was loaded isocratically in the phosphate buffer and eluted with a linearly increasing gradient of methanol from 0 to 15% over a 10 min interval followed by a constant level (15%) of methanol for the next 20 min. Detection was at 254nm.

5. Obtaining adducts of adenine and guanine

The adducts of the purines were made by exposing adenine or guanine as follows: Fifty ul of methylene chloride containing 0.5 mg of [^{14}C]BCES were transferred to a microfuge tube. One mg of the purine, dissolved in 1 ml of 0.01 N KOH containing 0.3%

NaCl and adjusted to pH 7.4, was added to the microfuge tube. The mixture was incubated at 37°C for 30 min. The solution was cooled to room temperature and applied to the HPLC column. HPLC was carried out on a Varian Model 5000 HPLC apparatus (Varian, Palo Alto, CA) using the following system: Elution system - 50 mM KH_2PO_4, pH 4.4 and absolute methanol (HPLC grade); Column - reversed phase, C_{18} ODS 2, Spherisorb (Phase Separations, Norwalk CT); Temperature - ambient; Flow rate - 1 ml/min; Program - sample was loaded isocratically in the PBS and eluted with a linearly increasing gradient of methanol from 0 to 15% over the period of 6-10 min followed by a constant level (15%) of methanol for the next 10 min. Detection was at 254nm.

6. Determination of total NAD

Total NAD in cultures was determined as follows: After the medium was removed and the cultures were washed with PBS, the dishes containing the cultures were put on a block of dry ice for 3 min. Then the culture dishes (22 mm) were put at about 3°C and the cultures were extracted two times with 0.33 ml of ice cold 0.1 M NaOH containing 1 mM nicotinamide. The extract was mixed well and neutralized to pH 7.2 with 0.1 ml of ice cold 0.37 M H_3PO_4. Total time in alkali was less than 2 min. After centrifugation at 13,000xg for 5 min, 0.76 ml of the supernatant solution was mixed with 10 ul of 20 mM phenazine ethosulfate and the solution was kept in the dark for 15 min while all the NADH was converted to NAD^+. The total NAD was then assayed as NAD^+ by the cycling assay as follows: The cycling assay was carried out as described by Bernofsky and Swan (1973) (also cf., Jacobson and Jacobson, 1976). This technique involved measuring the non-enzymatic reduction of thiazolium blue by NADH formed from NAD^+ by the oxidation of ethyl alcohol catalyzed by alcohol dehydrogenase. The reduction of the dye in 30 min determined by absorbance at 570nm was proportional to the amount of NAD^+ present in the reaction mixture. The reaction was standardized to the absolute amount of NAD^+ which was determined by spectrophotometric assay at 260nm and confirmed by reduction to NADH when alcohol dehydrogenase catalyzed the oxidation of alcohol. NADH was determined spectrophotometrically at 340nm.

An experiment was done to determine the recoveries of NAD^+ and NADH through the entire analytical procedure. Keratinocytes were isolated from the skin of newborn rats and divided into three equal samples. Ten nanomoles of NADH were added to one sample and 20 nmoles of NAD^+ to another. The third sample remained as the control. The three samples were extracted as usual with ice-cold 0.1 N NaOH containing 1 mM nicotinamide and neutralized with 0.37 M H_3PO_4 within 2 min. The analytical procedure described above was then followed. The amount of NAD^+ determined in the control sample was subtracted from the amounts of NAD^+ determined in the samples to which NAD^+ or NADH had been added to obtain the recovery of the added pyridine nucleotide. The recovery of NAD^+ was found to be 107% and that of NADH, 98%.

7. Assay of interleukin-1α (il-1α)

Each well in 96-well plates was coated with a monoclonal anti-human IL-1a by addition of 100 ul of 0.05 M carbonate-bicarbonate buffer, pH 9.6, containing 1`.5 ug of the antibody/ml and incubation overnight in the cold. The plates were then coated with bovin serum albumin (BSA) to minimize non-specific binding. After extensive washing with phosphate buffered saline (PBS) containing 0.05% Tween 20, 100 ul of a cell suspension were added to the well and the plate was incubated at 37° C for 60 min. The plate was washed, 100 ul of polyclonal rabbit anti-human IL-1α antibody were added and the plate was incubated for 60 min at 37° C. Then the plate was washed and 100 ul of peroxidase-conjugated goat amti-rabbit IgG were added. Finally, after incubation at 37° C for 30 min and washing, 100 ul of peroxidase substrate were added to each well. After 10 min, the reaction was stopped by the addition of 50 ul of 2.5 M sulfuric acid and the absorbance at 450nm was determined using a Titertek Multiscanner.

8. Assay of interstrand cross-linking in DNA

DNA interstrand cross-linking was determined using the ethidium bromide fluorescence technique described by Brent (1984). Two ml of a solution of ethidium bromide (1ug/ml) in 0.02 M potassium phosphate, pH 12, and containing 0.002 M EDTA were added to about 100 ul of colution containing 10 ug of DNA. Fluorescence was measured at 600nm with excitation at 525nm prior to and following denaturation. DNA was denatured by heating for 8 min in a boiuling water bath followed by rapid cooling to room temperature (i.e., within 10 min). In control samples of DNA, fluorescencewas nearly completely lost as a result of denaturation. After exposure to BCES, the presence of cross-links allowed the DNA to renatgure even though the sample was cooled rapidly and fluorescence was retained. The percentage of cross-linking in the DNA was calculated according to Garcia, et al. (1988). The amount of DNA was measured using the "DABA" assay of Setaro and Morley (1976).

9. Synchronization of cells in submerged cultures of keratinocytes from newborn rat skin.

Cells were synchronized using aphidicolin (Aph) by a modification of the method described by Matherly, et al. (1989). Aph is a reversible inhibitor of DNA polymerase and can be used to synchronize cells at the G1/S border of the cell cycle. At 36 hr after seeding, cultures were incubated with 1.5 ml of 1 ug of Aph/ml of medium at 35° C for 13 hr. Aph was then removed from the culture by washing with fresh medium once. After adding fresh medium, cultures were incubated for 9 hr and again exposed to Aph, this time for 14 hr at 1 ug of Aph/ml and 35° C.

10. Flow cytometric analysis of a cell's position in the cell cycle.

Cell suspensions in 0.5 ml of 70% ethanol were incubated at room temperature for 10 min and then stored at -20° C for 2-7 days. The following procedure was initiated 24 hr

before the flow cytometric analysis. Cells were sedimented by centrifugation and washed with PBS after which they were resuspended in 0.5 ml of 1% Triton X-100 and incubated at room temperature for 30 min. The cells were sedimented and incubated with 0.5ml of a solution of RNase(55 ug/ml) at 37^{o} C for 30 min. After centrifugation, cells were stained by the addition of 0.5 ml of propidium bromide (50 ug/ml) for at least 30 min. The samples were kept at 4^{o} C in the dark for no more than 24 hr.The samples were analyzed using an Epics Elite Flow Cytometer (Coulter Electronics, Hialeah, FL). Propidium bromide intercalates between base pairs of double-stranded DNA resulting in fluorescence. Excitation was done at 488nm with a 15-mwatt Argon ion laser. Filter combinations for measuring red linear fluorescence were a 488nm dichroic long-pass lens and a 630 nm band-pass filter. At least 2.5 X 10^4 single cells were accumulated at a flow rate of about 250 cells/sec. The area and peak values were recorded as a bivariate histogram and used to eliminate clumped cells from analysis. The ratio of area to peak discriminated between artifacts caused by doublets of G0/G1 cells and real single G2/M cells when intact cells were used (Bauer and Boezeman, 1983)The single parameter cell cycle distribution based on DNA content was stored using the Coulter Elite software and analyzed on an IBM AT compatible computer using the ModFit software package (Verity Software House, Topsham, ME).

C. Results

Results obtained during the period from 1 June 1990 to 31 October 1994 will be presented and discussed in context of the specific tasks established for this project .

Task 1: To establish the half-life of BCES in the aqueous medium to be used for exposing cultures or isolated cells to the mustard.

The objective of this task was to establish an order of magnitude for the time by which 1/2 of the BCES was hydrolyzed when incubated in PBS at 37°C which were the conditions used for exposing submerged cell populations to the mustard. This information was necessary in order to know how long experiments should be carried out so that conclusions from different types of experiments could be integrated. It was not intended to determine the absolute half-life for the hydrolysis since other investigators (cf., Burrows, et al., 1985) had studied this question in a definitive manner and had indicated the difficulties in obtaining accurate values for this parameter.

Samples of BCES in methylene chloride were dissolved in 250 ul of PBS, incubated at 37° C and assayed after various intervals for the residual amount of mustard in the aqueous medium as described in Section B, 1.

Figure 1 shows the absorbance at 560nm of samples taken from the $CHCl_3$ extract. The data indicate that half of the mustard was hydrolyzed in 21-22 min. This value approximates the value of 24 min reported for the half-life of mustard in 0.14 M NaCl at about 24°C and the value of 25 min in sea water at 30°C (cf., Papirmeister, et al., 1991).

Figure 2 shows the kinetics of disappearance of BCES from the solution when BCES in PBS was added to a submerged multilayered culture of keratinocytes in a culture dish and incubated at 37°C. The technique employed to determine the amount of BCES remaining in the solution, bathing the culture, was essentially the same technique used in the previous experiments in which $t_{1/2}$ was determined. The data indicated that half the BCES had disappeared from the medium by about 8 min after introduction into the culture dish. Presumably this time would vary depending upon the amount of the culture present, since the disappearance of BCES is a function of binding to cellular elements as well as hydrolysis *per se*.

No attempt was made to determine the kinetics of disappearance of BCES after application to the stratified cornified culture grown at the air-liquid interface because the quantitative recovery of the solution containing residual mustard from the surface of the culture would have been very difficult.

Figure 1. Decay of BCES in PBS at 37°C. The two curves represent the hydrolysis of two different concentrations of BCES. The $t_{1/2}$ was found to be 21-22 min. For procedural details, see the text. A_{560nm} of 10^2= 18 ug.

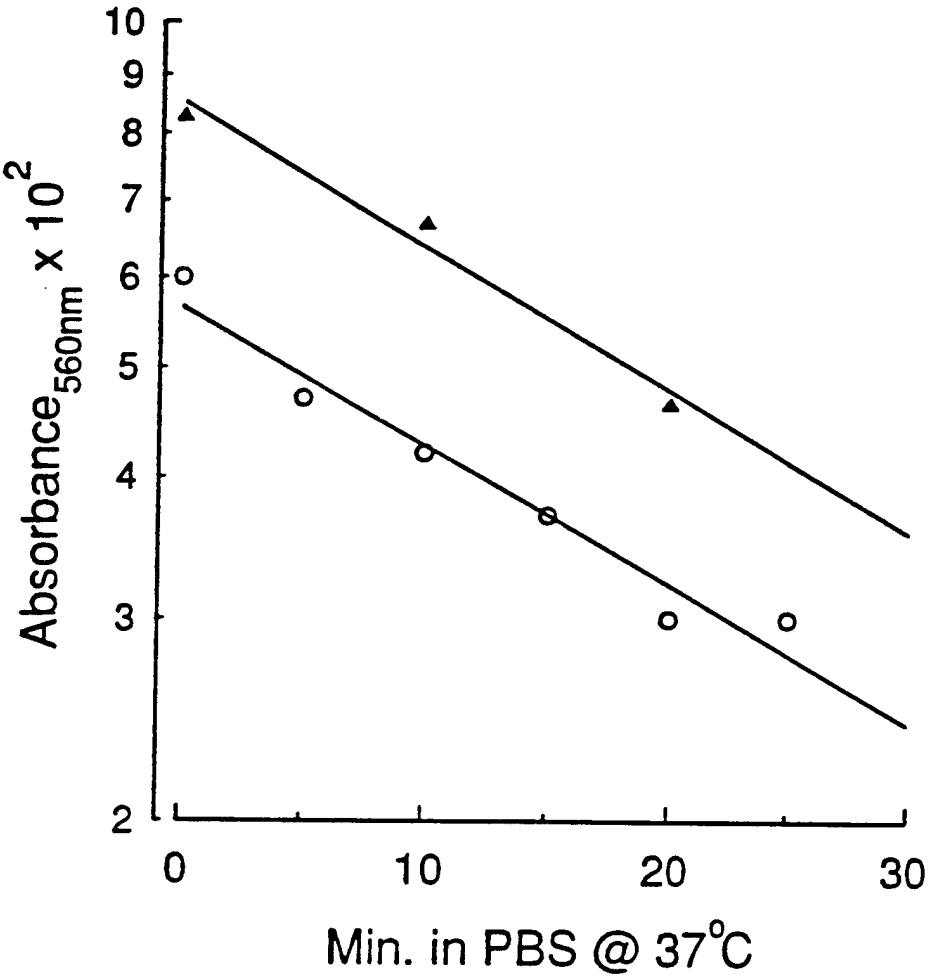
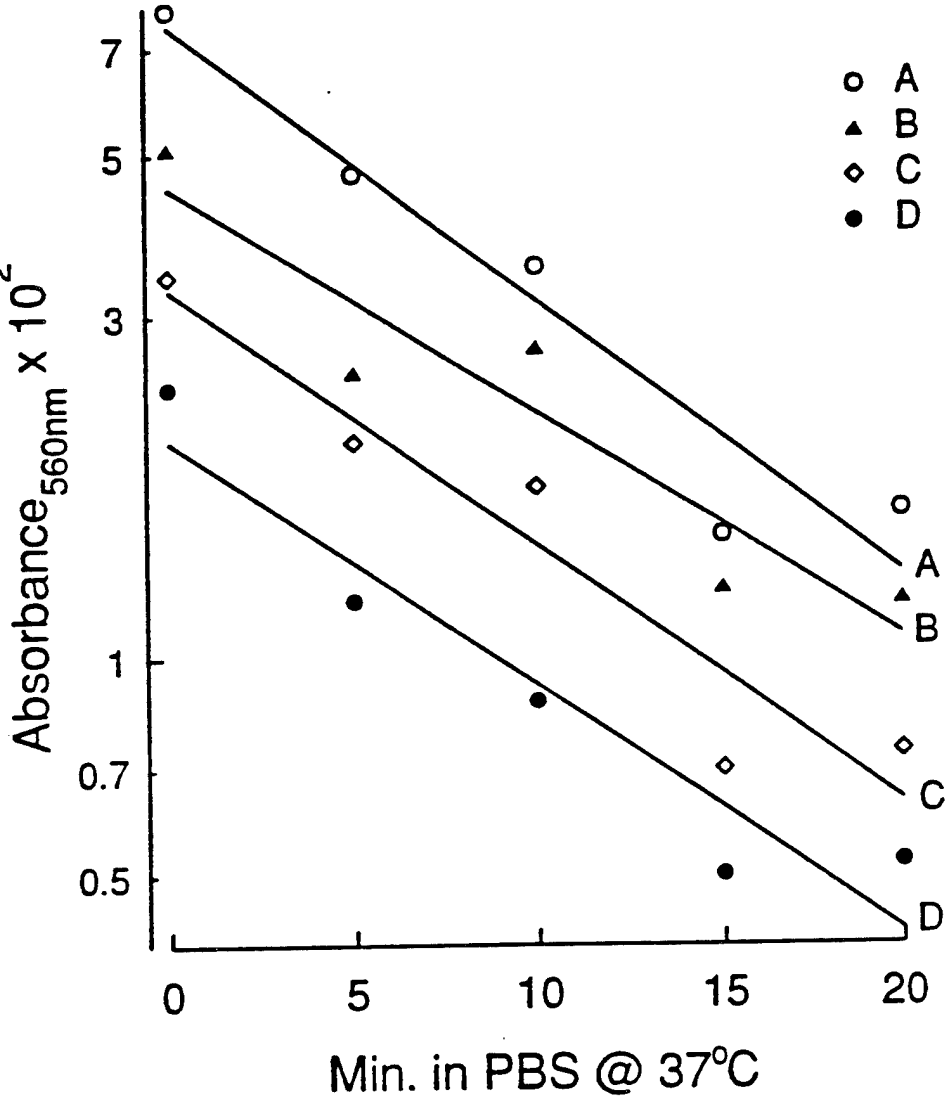


Figure 2. Disappearance of BCES in PBS when incubated at 37°C with a submerged multilayered culture of keratinocytes. Each of the curves represents the disappearance of BCES starting with a different concentration of the mustard. One-half of the BCES disappeared in about 8 min. For procedural details, see the text. A_{560nm} of 10^2= 18 ug. (A, 14 ug; B, 10 ug; C, 6 ug; D, 4 ug.)



Conclusion: The data obtained in this facet of the project indicated that by 30 min after addition of BCES to a submerged culture, less than 10% of the initial mustard remained. Therefore, 30 min should be the maximal time of exposure in such experiments. In reporting the level of exposure, doses of BCES can be reported as the amount initially added with a note calling attention to the rate of disappearance of BCES as defined by the results obtained in the work on Task 1 (i.e., 50% of the BCES disappears from the medium in about 8 min).

Task 2: To establish for human keratinocytes in culture, as was done for rat keratinocytes in culture, that DNA damage, repair, and informational error could be primarily responsible for initiating pathogenicity from BCES by

(a) Demonstrating in pseudo-epidermis that the incorporation of [^{3}H]TdR into DNA is inhibited earlier and after exposure to a lower level of BCES than the incorporation of [^{3}H]UR or [^{14}C]leu into RNA or protein, respectively.

Included under this task, was the demonstration that in cultures of human keratinocytes, as in cultures of rodent cells, the biosynthesis of DNA was more sensitive to BCES than was the biosynthesis of RNA or protein. Vaughan, et al. (1988) showed that in stratified, cornified cultures of rodent keratinocytes, the incorporation of [^{3}H]TdR into DNA was affected by exposure to a lower concentration of BCES than was the incorporation of [^{3}H]UdR into RNA or [^{14}C]leu into protein. Furthermore, the effect on DNA synthesis was seen earlier than was true for the other two synthetic processes.

Tables 1 and 2 show the effects of topical exposure of human pseudo-epidermis to various low doses of BCES on the synthesis of DNA and protein as monitored by the incorporation of [^{3}H]TdR and [^{14}C]leu, respectively. The BCES was applied in PBS for 30 min at 37^o C. Data are presented in terms of percentage of control. A value of 100% was assigned to the incorporation of the tracer after the culture was exposed to the vehicle (PBS) alone. The data confirm that the inhibitory effect of BCES on the incorporation of [^{3}H]TdR occurred at a lower dose than was necessary to obtain inhibition of [^{14}C]leu.

A dose of 0.56 nmol/cm^2 caused a 46% inhibition of the incorporation of [^{3}H]TdR when measured immediately post-exposure but a dose of 2.63 nmol/cm^2 was necessary to achieve a significant reduction in the incorporation of [^{14}C]leu at the same time. At a dose of 1.13 nmol/cm^2, inhibition of the incorporation of [^{3}H]TdR was 64% at 24 hr post-exposure and had not returned to control even by 48 hr post-exposure. However, by 48 hr post-exposure to 2.63 nmol/cm^2, the incorporation of [^{14}C]leu was normal.

The effect of exposure to low doses of BCES on the incorporation of labeled uridine into RNA was stimulatory. Immediately after exposure to 2.5 nmol/cm^2, the incorporation of [^{3}H]UR was 175% of control and was even 160% after exposure to 3.8 nmol/cm^2. No significant change from control was seen between 0.25 and 0.5 nmol/cm^2. As noted in a quarterly report in 1992, the effect of BCES on RNA synthesis was considered too

complex to study further given the time available and the purpose of this task. A similar situation was seen in pseudo-epidermis derived from rat cutaneous keratinoctes (cf., Vaughan, et al., 1988).

Table 1. Incorporation relative to unexposed control of [^{3}H]TdR into DNA of rat [seudo-epidermis at 0, 24 or 48 hr after topical exposure to BCES. Cultures were exposed to BCES in 50 ul of PBS for 30 min by topical application, washed with MEM three times and further incubated at 37° C in MEM. During the last 4 hr prior to harvest, 2 uCi of [^{3}H]TdR were added to medium. After labeling, cultures were washed with MEM and treated with 10% trichloroacetic acid. The resulting insoluble material (including the DNA and protein) was solubilized in 0.25 N NaOH after removal of the overlaying solution and counted in a liquid scintillation counter. Data are shown as percent of control value for incorporation of isotopic precursor.

nmol/cm^2	Hr post-exposure		
	0	24	48
0 (PBS Control)	100 n=5	100 n=6	100 n=11
0.19	86±8 n=5	94±21 n=6	103±26 n=8
0.56	54±10 n=8	61±12 n=6	96±33 n=10
1.13	54±13 n=6	36±29 n=7	75±24 n=11
1.88	23±6 n=6	12±6 n=10	55±17 n=8

Table 2. Incorporation of [^{14}C]leu into protein in rat pseudo-epidermis at 0, 24 or 48 hr after topical exposure to BCES relative to unexposed controls. For technical details, see the legend to Table 1. 1.5 uCi of [^{14}C]leu was added to medium for labeling the cells during the last 4 hr prior to harvesting. Data are shown relative to control value for incorporation of labeled precursor.

nmol/cm^2	Hr post-exposure		
	0	24	48
0 (PBS control)	100 n+8	100 n=8	100 n=8
1.13	97±16 n=8	97±16 n=8	111±16 n=10
1.88	89±10 n=8	89±22 n=8	109±13 n=11
2.63	62±17 n=8	68±15 n=8	103±14 n=11

Conclusion: In pseudo-epidermis, the incorporation of [^{3}H]TdR - a measure of DNA synthesis - is more sensitive to inhibition by mustard than is the incorporation of [^{14}C]leu - a measure of protein synthesis. Furthermore, protein synthesis shows an earlier recovery than does the synthesis of DNA. The effects of BCES on the synthesis of DNA, RNA and protein are similar in pseudo-epidermis derived from cutaneous keratinocytes of the rat and the human. DNA synthesis - principally replication that occurs in the basal germinative population of pseudo-epidermis - is clearly a very sensitive target for BCES in pseudo-epidermis. In the human skin, structural damage to DNA resulting from alkylation by BCES has been postulated to be the initiating event in vesication and inhibition of DNA synthesis is a known concomitant of the process *in vivo*.

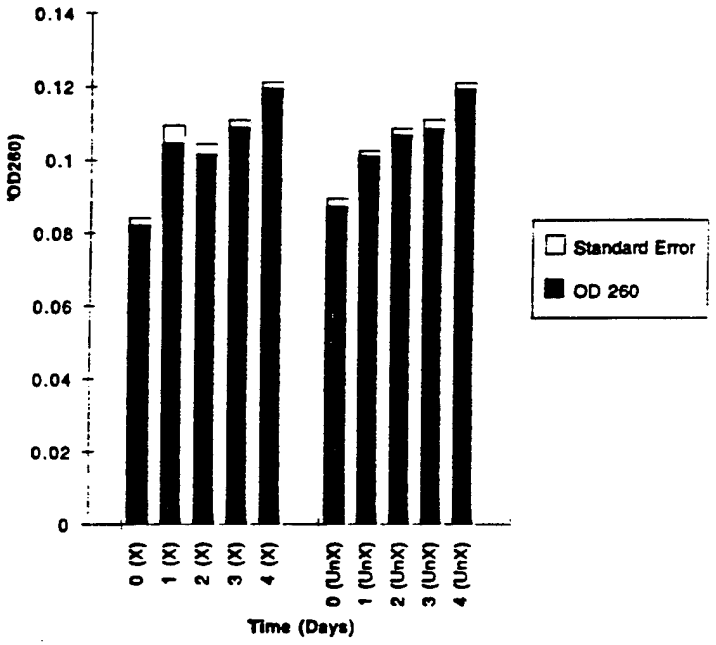
(b) Demonstrating in submerged cultures that although gross structural integrity of DNA can be restored to normal, DNA replication remains inhibited for a period thereafter, and the morphology of the culture undergoes a change.

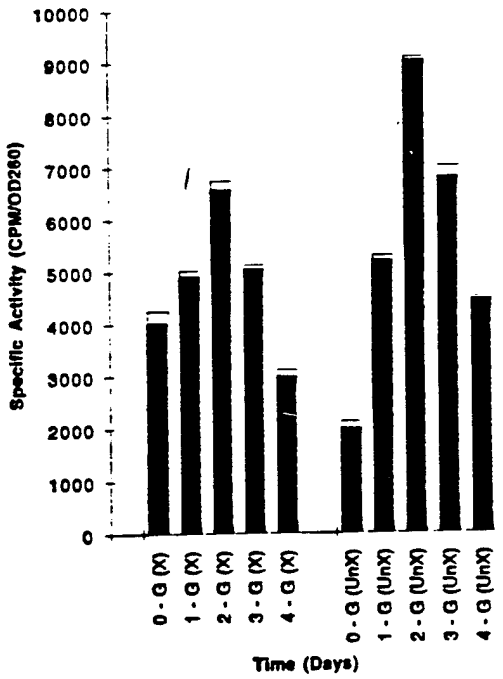
Ribeiro, et al reported in 1991 that the single-strand breaks found in the DNA of monolayer cultures of rat keratinocytes immediately after exposure to as much as 5 uM BCES disappeared during 22 hr of subsequent incubation. Complete restoration of the structural integrity of its DNA appeared to have been accomplished. Nevertheless, Ku and Bernstein (1988) found that cultures exposed to 5 uM BCES lost a major fraction of their cells at 7 days post-exposure and did not resume DNA replication. In fact a substantial portion of the remaining cells showed the specific lectin-binding that is characteristic of differentiated cells. Even in cultures exposed to 1 uM BCES, abnormally large differentiated cells were present at 7 days post-exposure.

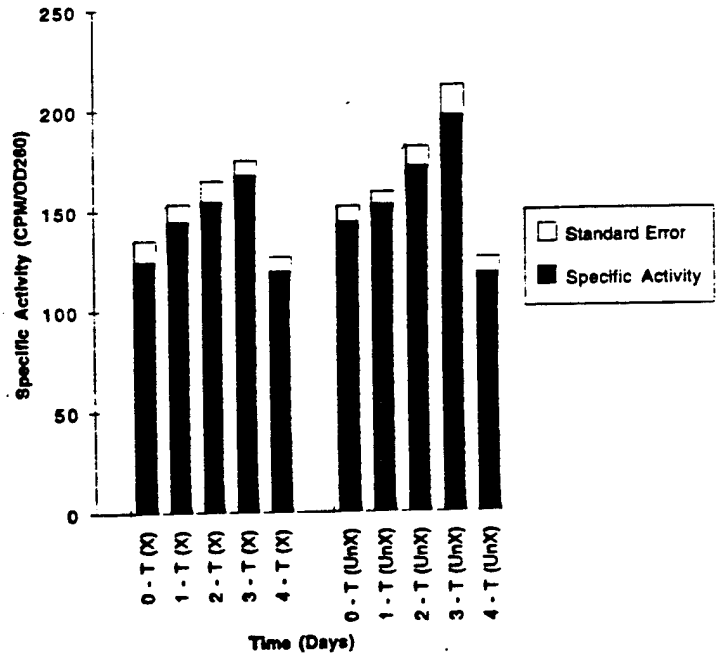
To check whether or not exposure to 1 uM BCES would cause a similar inhibition of DNA replication in cultures of human keratinocytes, confluent cultures were exposed to BCES for 30 min and then pulse-labeled with [^{3}H]TdR or [^{3}H]GR for 1 hr or were incubated in fresh growth medium for 1, 2, 3 or 4 days and then pulse-labeled in the same manner Figure 3A shows the total absorbance$_{260nm}$, a measure of the total number of cells present, at 0, 1, 2, 3 or 4 days after cultures were exposed to 1 uM BCES. Also shown in this figure is the total absorbance of similar cultures that were not exposed to the mustard. The data show that both the treated and untreated cultures showed a 50% increase in absorbance over the period of 4 days. In spite of the similarity in expansion of the cultures, Figure 3B and 3C show that the amounts of [^{3}H]TdR and [^{3}H]GR that were incorporated into the DNA per unit of absorbance over the same period were similar in pattern but different in quantity. The incorporation of [^{3}H]TdR per unit of absorbance (i.e., the specific activity) was significantly higher in the untreated cultures over the first 3 days This effect was even more dramatic when [^{3}H]GR was used to monitor DNA replication. In cultures that were exposed to BCES, as expected from data reported in Task 5, Section b), the incorporation of [^{3}H]GR was greater immediately after exposure than it was in the untreated culture at the same time.

Conclusion: Since the total amounts and the patterns of change in the DNA (based upon the total absorbance $_{260nm}$) were similar in the treated and untreated cultures, it is reasonable to assume that exposure to BCES did not significantly alter the total number of cells present in the cultures over the entire experimental period. Also, except for the incorporation of [3H]GR immediately after exposure, the patterns of specific radioactivity in the DNA resulting from the incorporation of both tracers were not significantly different. In fact, only the levels of specific radioactivity were different in the treated and untreated cultures being significantly higher in the latter cultures. A reasonable explanation for these observations is that the number of cells that synthesized DNA was lower in the exposed as compared with the untreated cells. In submerged monolayer cultures of rat cells, exposure to 1 uM BCES resulted in a 30% increase in the percentage of differentiated cells (i.e., cells that do not synthesize DNA and are committed to programmed cell death) (Ku and Bernstein, 1988). The data obtained in this sub-task allow the conclusion that, as in cultures of rat

Figure 3. Amounts and synthesis of DNA, in control submerged cultures of human keratinocytes and in cultures exposed to 1 uM BCES, immediately after exposure and 1, 2, 3 or 4 days later. Confluent cultures were exposed to BCES for 30 min, washed to remove the mustard and further incubated. Assay for total absorbance$_{260nm}$ and incorporation of [^{3}H]TdR or [^{3}H]GR by pulse-labeling of the DNA for 1 hr were carried out over a period of 4 days. For other details, see Section B, above.







keratinocytes, exposure to low levels of BCES results in a decrease in the germinative fraction and an increase in the differentiated fraction of cells in the culture.This effect could be a consequence of an informational change in the DNA.

(c) Demonstrating in pseudo-epidermis that basal cells become necrotic after a lower dose of topically applied BCES and sooner after exposure than differentiated cells.

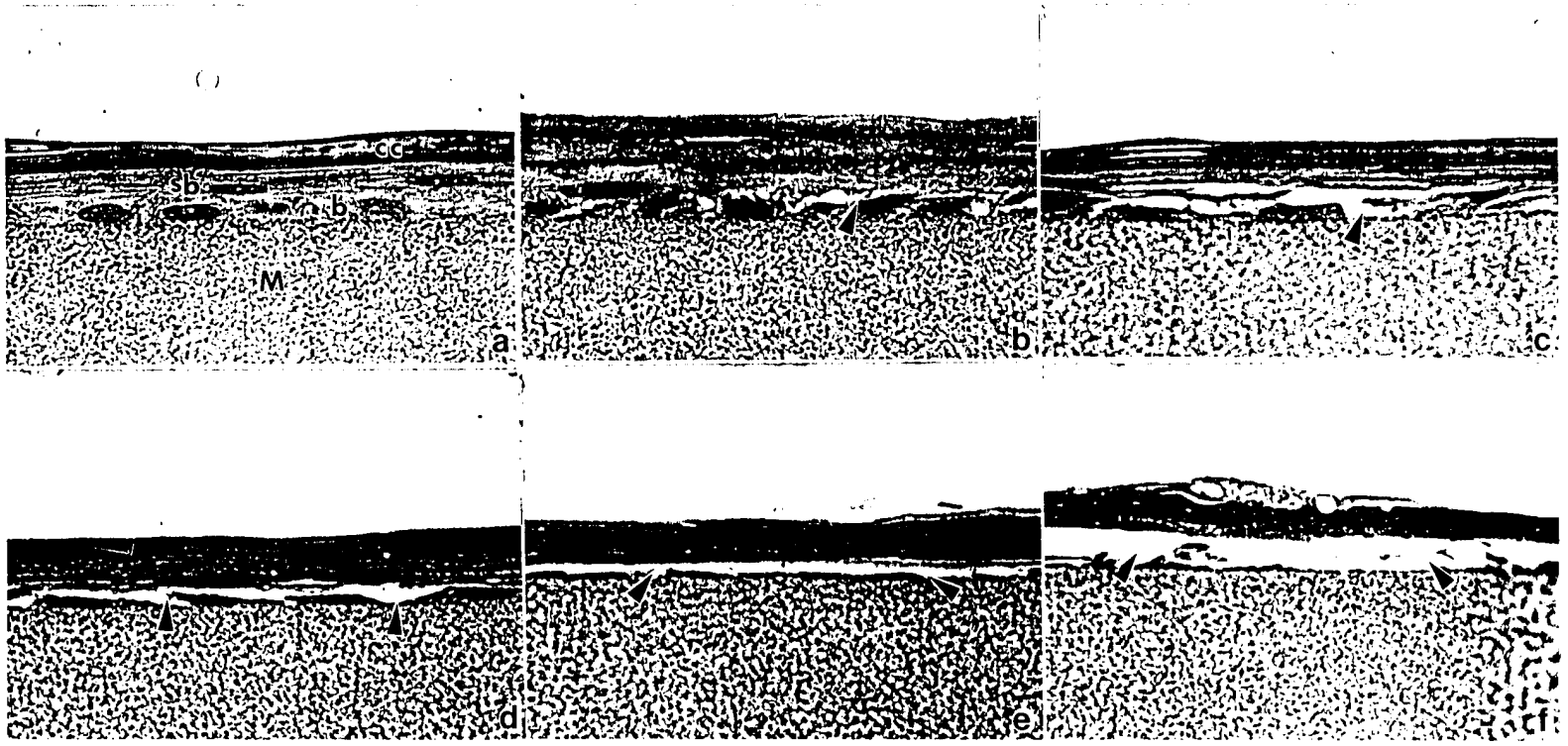
As previously noted (cf., Section A), vesication in skin after topical exposure to BCES initially involves the loss of the epidermal basal and lower spinous layers. This observation indicates that the population of basal cells is more sensitive to BCES than are the majority of the differentiated cells. This sub-task was designed to determine whether the human pseudo-epidermis would exhibit a similar phenomenon after exposure to BCES as had been shown to occur in pseudo-epidermis derived from rat keratinocytes (cf., Bernstein, et al., 1987).

Cultures of human keratinocytes grown submerged for 4 days and lifted for 21 days were topically exposed to 25, 50 or 100 nmol of BCES (in PBS) per cm^2 of surface area. Control cultures were exposed to PBS. Cultures were washed, further incubated in growth medium for 24, 48 or 72 hr at 37° C in fresh growth medium. fixed in neutral buffered formaldehyde and embedded in paraffin. Sections were prepared, stained with H and E and observed by light microscopy.

Figure 4 presents photomicrographs of cross sections taken from human pseudo-epidermis exposed in such experiments. After the lowest level of exposure to BCES, no morphological changes from control were evident at 24 hr post-exposure (Fig. 4a). At 48 hr post-exposure to this level of BCES, a few degenerated cells were seen in the basal layer (Fig. 4b). At 72 hr post-exposure there were foci of degeneration in the basal layer (Fig. 4c) After exposure to 50 nmol/cm^2, areas of degeneration were visible in the basal layer at 24 hr post-exposure (Fig. 4d). At 48 hr post-exposure to 50 nmol/cm^2, the entire basal layer had disappeared (Fig 4e). In the case of the highest level of exposure, the basal layer exhibited extensive degeneration at 24 hr post-exposure (Fig 4f). At this time, degeneration involved the lower suprabasal cells, as well.

Conclusion: Topical exposure of human pseudo-epidermis to between 25 and 100 nmol of BCES/cm^2 results in a graded loss of the basal layer after subsequent incubation for 24-48 hr.The differentiated layers remain intact and show minimal morphological change. These results establish the level of exposure and time of incubation after exposure necessary to bring about destruction of the basal layer in human pseudo-epidermis - a surrogate for the loss of the basal layer as a result of the vesicant action of BCES *in situ*. This level is two orders of magnitude greater than the level (between 0.19 and 0.56 nmol/cm2) at which an inhibition of DNA synthesis - a likely indicator of the initial interaction between BCES and the skin - becomes evident.

Figure 4. Cross-sectional light photomicrographs of stratified, cornified cultures of human keratinocytes at 24, 48 or 72 hr post-exposure to 0, 25, 50 or 100 uM BCES/cm^2. For procedural details, see the text. Level of exposure: a - Solvent control (PBS only) or 25 umol/cm^2; b, c - 25 uM BCES/cm^2; d, e - 50 uM BCES/cm^2; f - 100 uM BCES/cm^2. Incubation time post-exposure: a, d, f - 24 hr; b, e - 48 hr; c - 72 hr. Arrows indicate areas of degeneration. Magnification X592



Task 3: To identify the entities which are the major mono-functional and bifunctional (i.e., cross-linked) alkylation sites in the DNA of cultures of human keratinocytes exposed to low levels of BCES and determine whether the DNA in the basal and differentiated cells have different profiles of adducts by

(a) Exposing [^{3}H]DNA, isolated from cultured keratinocytes grown in the presence of tritiated nucleosides, to [^{14}C]BCES at pH 7 in order to establish the technology for identifying and quantifying the adducts that can occur in the DNA of cells exposed to sulfur mustard.

In order (a) to identify the adduct(s) in DNA which are formed when keratinocytes are exposed to low levels of BCES, (b) to determine which adduct(s) correlate with loss of viability in the germinative population of keratinocytes (as was planned for Task 5, cf., below), and (c) to compare the profile of adducts present in proliferative and differentiated keratinocytes, it was deemed necessary to have an assay for the alkylation products that was more sensitive than ultraviolet absorbance. It was felt that using absorbance as an assay would require too large a number of cultures. Radioactive tracer technology coupled with HPLC seemed the experimental approach of choice in this regard. Furthermore, having the cellular DNA labeled specifically in the guanine, adenine, cytosine or thymine moiety, by growing cells in the presence of the appropriate radioactive precursor of DNA, would make it more convenient to identify an adduct of a particular base. To this end, DNA, labeled with ^{3}H in the guanine moiety (as shown by hydrolyzing an aliquot of the [^{3}H]DNA for 60 min in 1N $HClO_4$ at 100^o C and finding guanine as the only labeled base by HPLC), was prepared by growing keratinocytes in the presence of [^{3}H]GR. This [^{3}H]DNA was then exposed to various concentrations of BCES or [^{14}C]BCES, hydrolyzed to liberate the alkylated bases, and submitted to gel filtration on a column of BioRad P2 to separate the residual DNA from the liberated adducts. The adduct fraction was then resolved on a C18 reverse phase HPLC column.

Initial efforts to identify and quantify BCES-mediated adducts in [^{3}H]DNA were done with DNA isolated from cultures of keratinocytes that were derived from rat skin. In the procedure used, a sample of [^{3}H]DNA was exposed to [^{14}C]BCES; the sample was then heated in a boiling water bath for 30 min at neutral pH to hydrolyze the glycosidic linkage between alkylated bases and the deoxyribose; the residual DNA was removed by gel filtration, and the eluted fractions were submitted to HPLC for identification and quantification of the alkylation products. If neutral thermal hydrolysis of the linkage between normal bases and the deoxysugar were to occur to some small extent, this was not considered to be a major problem since alkylated and normal bases were expected to be separable on HPLC.

Figure 5 indicates the separation achieved by BioRad P2 between unreacted DNA, which appeared at the V_o, i.e., at the exclusion volume of the column, and free guanine which marked the V_i, i.e., the imbibed volumn of the column. Peak A, the position of the DNA, was adequately separated from Peak B, guanine, at a rate of flow that was 1 ml/min. Figure 6 shows the elution profiles of absorbance$_{254nm}$, ^3H and ^{14}C which were obtained when 0.5 mg of [^3H]DNA, that was labeled in the guanine residues, was exposed to 2.75 mM [^{14}C]BCES in PBS for 30 min at 37° C, then thermally hydrolyzed at neutral pH and submitted to gel filtration on a column of Bio-Rad P2. The profile of ^3H from the column indicates that a substantial fraction of all the guanine residues in the DNA had been hydrolyzed from the polymer. About 20% of the ^3H put on the column remained with the polymer at the V_o. However, the hydrolyzed guanine residues, based upon ^3H, appeared at a position in the elution profile that was intermediate between the positions of DNA and guanine. The only peak of ^{14}C appeared in the same fractions as did the peak of ^3H although the two peaks were not quite coincident. It became evident from this type of data that the column of BioRad P2 being used was not large enough to provide adequate resolution of the various substances in the hydrolysate of samples of alkylated DNA.

For further analysis of the doubly labeled peak, a larger column of BioRad P2 was utilized. This column was 20 cm X 1.4 cm^2 and had a matrix volume of 27 ml. The column was sufficiently large to easily separate the residual DNA from free guanine. The V_o of this column was 12 ml (i.e., at Fraction 12) and the V_i was determined to be 46 ml. The conditions for hydrolysis of DNA were also changed to maximize the hydrolysis of the alkylated base and minimize the hydrolysis of the normal base from their glycosidic linkages (cf., Ludlum, et al., 1994). Another change was the use of [^3H]DNA derived from cultures of human - rather than rat - keratinocytes. Figure 7 shows the gel permeation profiles of purified <u>untreated</u> [^3H]DNA before and after hydrolysis for 30 min at pH 3.5 and 70° C. Although a small amount of absorbance at 254_{nm} was observed in Fractions 15-26 whether the DNA was hydrolyzed or not, all the ^3H appeared in Fractions 11-15 in both cases. Clearly, the technique in use did not significantly hydrolyze the glycosidic linkage between normal guanine and the deoxysugar since there was no ^3H in Fraction 46.

Figure 8 presents the gel permeation profiles of ^3H and Figure 9 the profiles of ^{14}C that were obtained when samples of this [^3H]DNA were exposed to 50, 150, 300, 1000 or 5000 uM [^{14}C]BCES and hydrolyzed as noted above. Essentially all of the ^3H that was not excluded from the column (i.e., that did not appear as DNA in Fractions 11-14) appeared in a dose-responsive manner in one peak found in Fractions 24-32. In the case of the exposure to 50 uM BCES, 8% of the counts appeared in the second peak. After the exposure to 5000 uM BCES, 75% of the 3H was found in the second peak. A decrease in the amount of ^3H found at the position of DNA paralleled an increase in the ^3H eluted as adduct in Fractions 24-32. Only at the higher exposures was any radioactivity observed beyond Fraction 32.

Figure 5. Gel filtration of DNA (A) and free guanine (B) on a column of BioRad P2. Sample put on column in 0.1 mM phosphate, pH 4.4 and eluted with the same solution. Rate of flow 1ml/2 min. Column volume: 9 ml. Column dimensions: 9.5 cm X 0.9 cm^2. Fraction volume: 1 ml. Vo (exclusionm volume): 4 ml. Vi (imbibed volume): 10 ml.

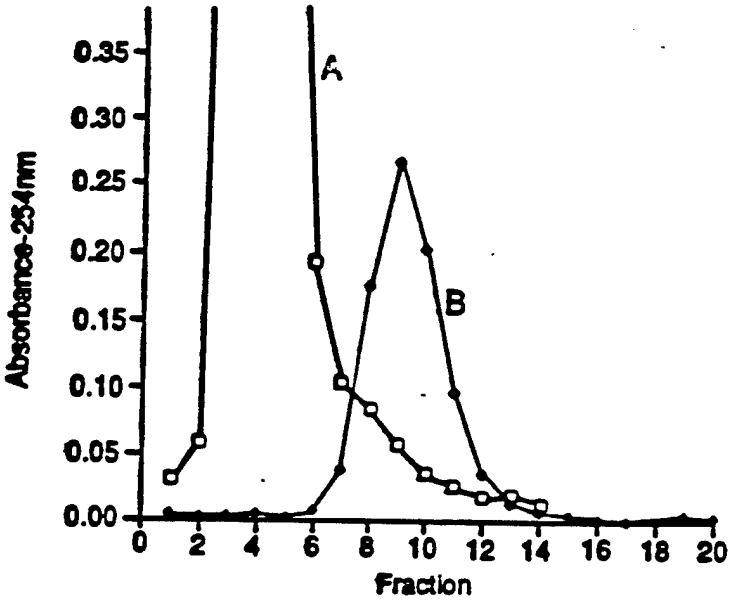


Figure 6. Profiles of absorbance at 254nm, ^{3}H and ^{14}C obtained when 0.5 mg of DNA labeled with ^{3}H in the guanine residues was exposed to 2.75 mM [^{14}C]BCES in 1 ml of PBS at 37°C for 30 min, thermally hydrolyzed in 0.01 M Tris-EDTA, pH 7, at 100° C and submitted to gel filtration on a column of Bio-Rad P2. For procedural details of the gel filtration, cf., legend for Figure 5. -□-, absorbance; -●-, ^{14}C and -△-, ^{3}H.

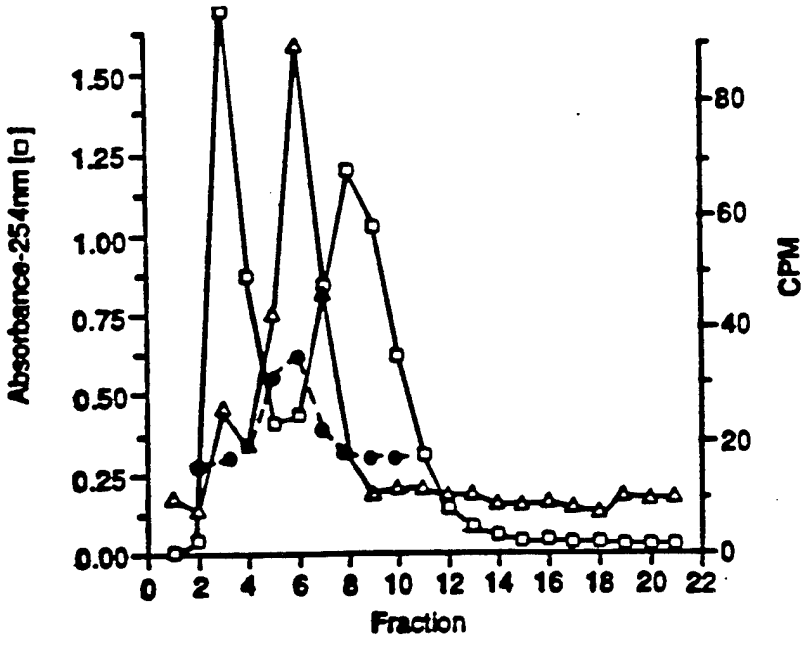


Figure 7. Gel permeation profile of hydrolyzed (A, C) and unhydrolyzed (B, D) samples of untreated DNA isolated from human keratinocytes labeled in the guanine residues by growth in the presence of [^{3}H]GR. Hydrolysis was carried out at pH 3.5 for 30 min at 70° C. Gel permeation was done on a column (20 cm X 1.4 cm^2) of BioRad P2 with V_o=12 ml (i.e., in Fraction 12 and V_i=46 ml (i.e., in Fraction 46). Recovery from the column was >80% in each run.

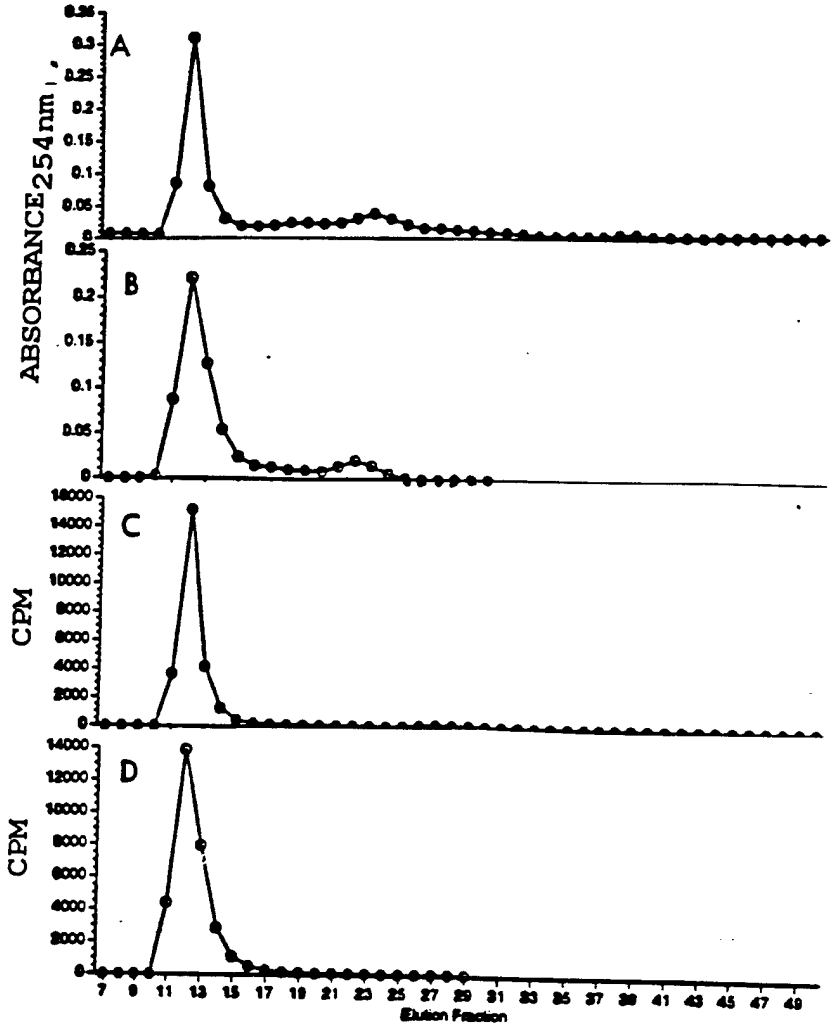


Figure 8. Gel permeation profile of samples of [^{3}H]DNA, derived from cultures of human keratinocytes grown in the presence of [^{3}H]guanosine, that had been exposed to 50, 150, 300, 1000 or 5000 uM [^{14}C]BCES and hydrolyzed. For additional technical details, see the legend for Figure 7. Figure 9 shows the ^{14}C labeling of these samples of DNA.

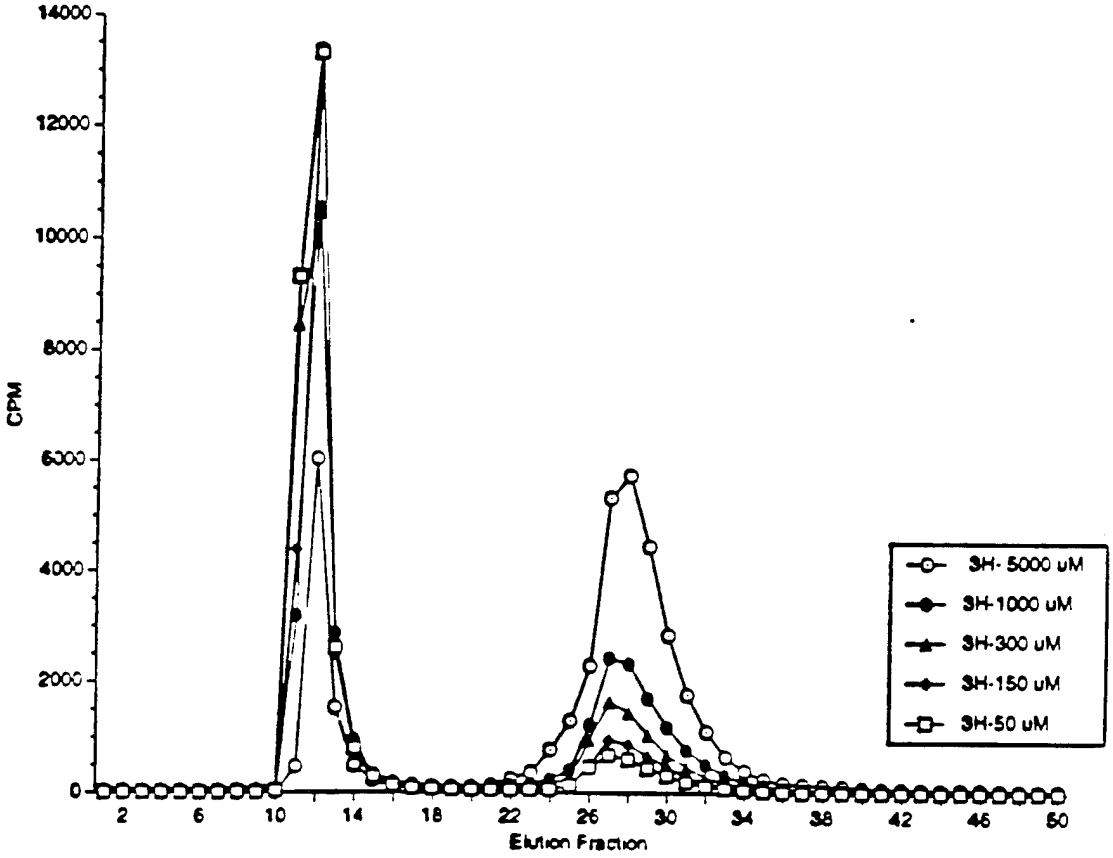
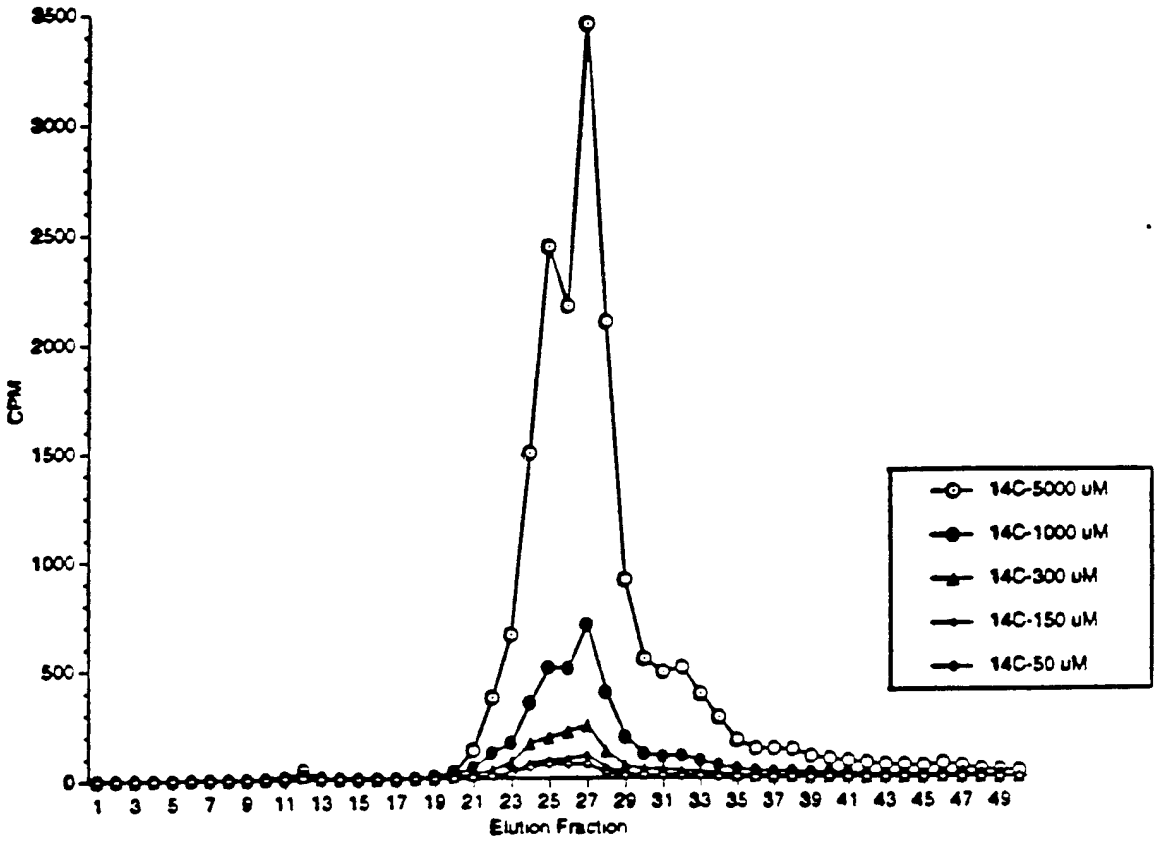


Figure 9. Gel permeation profile of ^{14}C from use of [^{14}C]BCES monitored in the same experiment as shown in Figure 8.



The profile of ^{14}C eluted from the column of BioRad P2 (Figure 9), indicates that Fractions 21-35 contained nearly all the 14C when samples of DNA that were exposed to 50, 150, 300 or 1000 uM BCES were hydrolyzed and gel filtered on the BioRad P2 column. In the analysis of DNA exposed to 5000 uM BCES, a small amount of ^{14}C also appeared in Fractions 35-47. Since using [^{14}C]BCES as a tracer, results in the labeling of all adducts - not just the guanyl-adducts - it is not surprising that the shape of the major peak of ^{14}C in the elution profile is not exactly the same as is shown for ^{3}H in Figure 8. However, the peak tube for each isotope was Fraction 27.

Based upon the shape of the elution curve for ^{14}C in Figure 9, at least four different substances were eluted between Fractions 21 and 41. Those that eluted in positions different from the single peak of ^{3}H, as shown in Figure 8, could be adducts of moieties of DNA other than guanine. The absence of any excluded ^{14}C in the profiles of Figure 9 indicates that the hydrolytic proedure in use liberated all the adducts of the DNA.

The literature indicates (cf., Section A, 2, d, above) that the N^7-mono-adduct of guanine is the most prevalent alkylation product in DNA after exposure to BCES. This was true in the case of DNA from a variety of species. The N^3-mono-adduct of adenine and the N^7-diguanyl-adduct are reported to be the next in quantity. The molecular weights of the N^7-mono-adduct of guanine and the N^7-di-guanyl adduct are 256 and 386, respectively. The molecular weight of a substance eluted from the BioRad P2 column in Fraction 27, the peak tube of ^{3}H shown in Figure 8, should be about 420, based upon the standardization of the column (cf., Fig. 10). From Figure 10, a molecule of molecular weight 256 should appear in Fraction 37. From these considerations, it appeared that, in human keratinocyte DNA exposed to BCES, the mono-adduct of guanine was not the major adduct and that the di-guanyl adduct had to be considered as an adduct present in large quantity.

In an effort to gain some additional information regarding the identify of the labeled substance(s) eluted from the column of BioRad P2 at the level of about 400 molecular weight, combined Fractions 25-29 of the eluates from the gel permeation of DNA exposed to 300, 1000 or 50000 uM BCES, were submitted to HPLC. Figure 11 shows the times at which authentic samples of bis-(2-guanin-7-yl-ethyl)sulfide (4 min), guanine (9 min) and 7-(2-hydroxyethylthioethyl)guanine (21 min) were eluted from the HPLC column. The authentic samples were monitored by absorbance at 254nm. The radioactivity was monitored downstream from the absorbance monitor by collecting fractions in a fraction collector. Standardization of the monitoring system with radioactive guanosine showed that radioactivity was observed about 1 min after absorbance was monitored. The stream was collected in fractions of 0.5 ml/0.5 min. Figure 12 shows the

Figure 10. Standardization of a BioRad P2 column for molecular weight. Matrix: 20 cm X 1.4 cm^2, 27 ml. Figures in parentheses indicate molecular weights of authentic samples of the marker compounds.

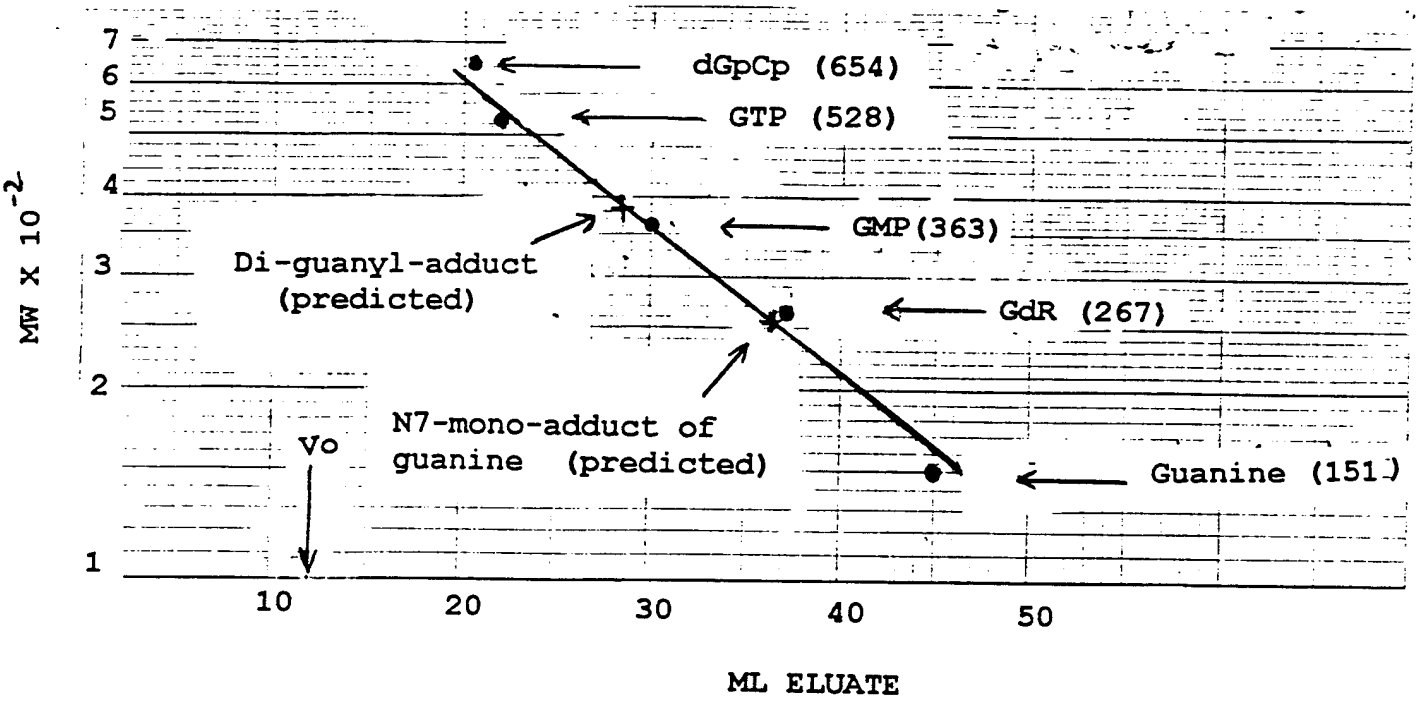


Figure 11. Position on HPLC of authentic samples of bis-(2-guanin-7-yl-ethyl)sulfide, guanine and 7-(2-hydroxyethylthioethyl)guanine. Absorbance monitored at 254nm.

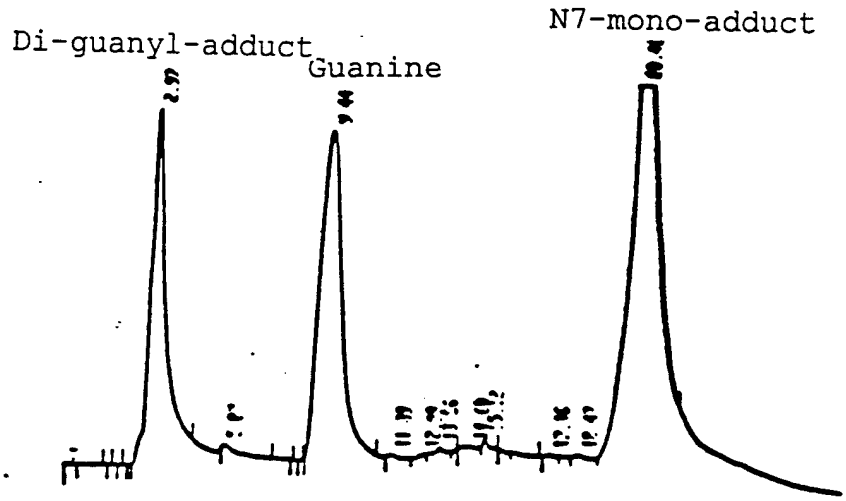
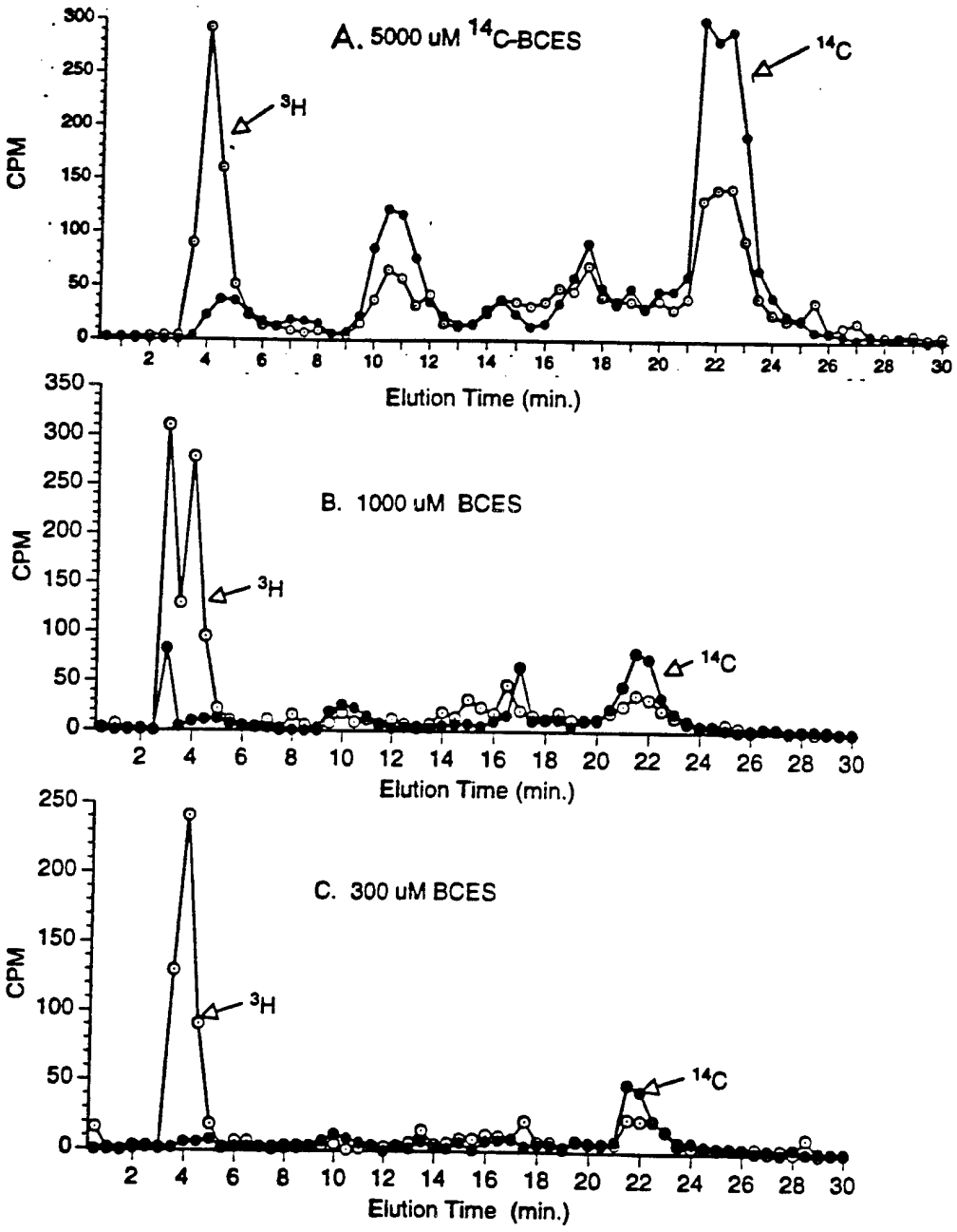


Figure 12. HPLC profiles of ^{3}H and ^{14}C from combined Fractions 25-29 derived from the gel permeations shown in Figures 8 and 9. Only the "300, 1000 and 5000 uM BCES samples were submitted to HPLC. For technical details, see Section B4, Methods.



HPLC elution profiles for combined Fractions 25-29 derived from the BioRad P2 column separations (cf., Fig. 8 and 9) of the DNA hydrolysates after exposure to 300, 1000 or 5000 uM BCES. In all three cases, a major peak of ^{3}H was eluted at 4 min, the position of the di-guanyl adduct. Based upon the assay of comparable aliquots during the exposure and analytical procedure, the amount of ^{3}H in the 4 min peak was the same at all three levels of exposure. On the other hand, lesser peaks at 10, 14, 17 and 21 increased as the dose of BCES was increased. The peak at 21 min, the position of the N^{7}-mono-adduct of guanine, was the largest of these minor peaks.

Surprisingly, although peaks of ^{14}C appeared to elute coincidentally with the minor peaks of ^{3}H, there was no comparable peak of ^{14}C at 4 min. This observation was replicated in a separate experiment at an exposure of 5000 uM BCES. The absence of ^{14}C in the peak at 4 min argues strongly against the di-guanyl- adduct being the identity of the major component of ^{3}H in this fraction.The elution of a small - but dose-responsive - amount of ^{14}C between 4 and 5 min, however, could indicate the presence of a small amount of the di-guanyl-adduct.

Two questions are raised by these data. Firstly, what is the identity of the peak eluting at 21 min by HPLC? Is it really the mono-adduct of guanine? If so, why does it appear to have a molecular weight of about 420 when gel-permeated on BioRad P2? No certain answers to these questions are presently available. It is possible that the gel permeation of the mono-adduct of guanine on BioRad P2 is influenced not only by the size of the molecule but also by hydrophobic binding. Secondly, does the peak at 4 min on HPLC have any toxicological relevance and, if so, what is the identity of the substance which contributes most of the ^{3}H to the peak? To be toxicologically relevant, it would seem that the size of the peak at 4 min should be dose-responsive. In a control experiment for the 5000 uM-exposure, in which mustard was omitted, a small peak of ^{3}H was present at 4 min, suggesting that exposure to BCES increased the amount of the ^{3}H that appeared at 4 min on HPLC but was not required to have the substance make an appearance. In the absence of evidence for a dose-related increase in quantity, it appears reasonable to ascribe the phenomenon to non-relevance with respect to the toxicology of BCES. Supporting this conclusion are the results of similar experiments done with [^{3}H]DNA, derived from rat keratinocyte cultures, and [^{14}C]BCES. Figure 13 shows the profile from a column of BioRad P2. Again, the major retarded peak of radioactivity appeared in Fractions 24-29. Fraction 27 from this profile was then submitted to HPLC by which the profile in Figure 14 was obtained. In this case, the major peak of ^{3}H and ^{14}C appeared at 21 min. No peak of ^{14}C was found at 4 min. Presumably, the ^{3}H-labeled substance eluting at 4 min on HPLC is peculiar to DNA from human - but not rat - keratinocytes

Conclusion: The earlier conclusion that the di-guanyl-adduct was the major adduct found when DNA from cultures of human keratinocytes were exposed to low levels of BCES is untenable. The N^{7}-mono-adduct of guanine seems to be the major adduct in this system as it is in other systems that have been studied. The profiles of tracer in Figure 12 indicate that the technology which has been developed can be used to identify and, possibly, quantify adducts. However, the relatively low level of ^{3}H and ^{14}C found after an exposure of

Figure 13. Gel permeation profile of [^{3}H]DNA, derived from rat keratinocytes, exposed to 300 uM [^{14}C]BCES, hydrolyzed and passed through a column of BioRad P2. For technical details, see the legend for Figure 7.

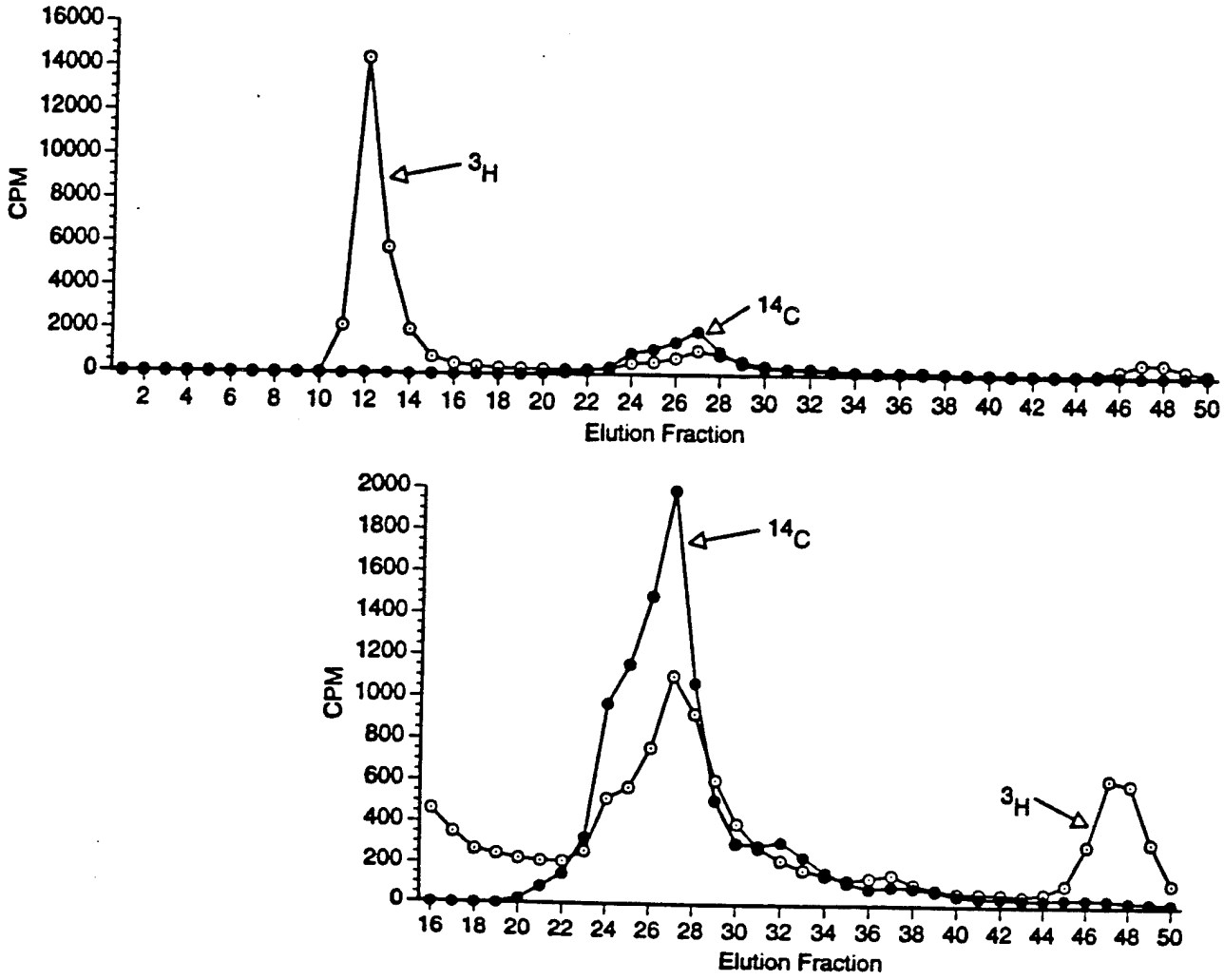
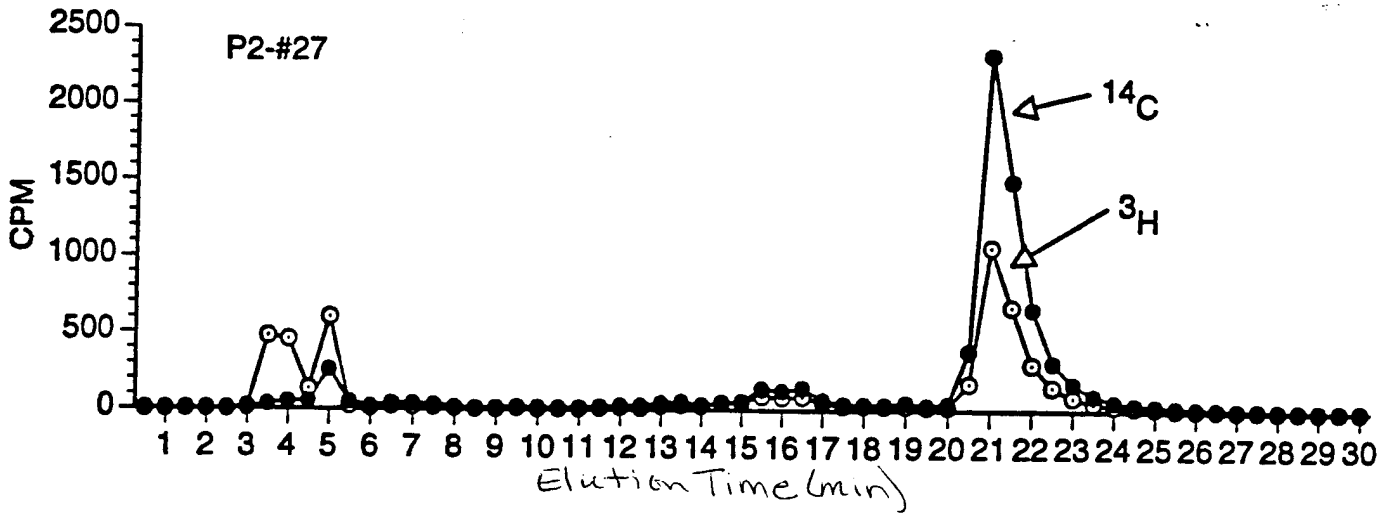


Figure 14. HPLC profile of ^{3}H and ^{14}C from Fraction 27 derived from the gel permeation shown in Figure 13. For technical details, see Section B, 4, above.



300 uM BCES made it clear that the analytical procedure would have to be modified to increase its sensitivity if it were to be successfully used at the low doses of mustard which produced cytotoxicity in cultures of keratinocytes.

(b) exposing monolayer cultures of human keratinocytes, that have previously been labeled with one of the four tritiated deoxynucleosides during growth, to 1 and 5 uM [^{14}C]BCES, respectively, and quantifying by HPLC the adducts present in each case immediately after exposure and at various times post-exposure.

Initial work on this sub-task, done at the beginning of the second half of the contract period, indicated that exposing cultures of keratinocytes to [^{14}C]BCES at the specific activity then available, did not label adducts at a sufficiently high level so that their formation could be monitored and their identity could be established. Therefore, it was decided to grow cells in the presence of [^{3}H]GR in order to label their guanine moieties so that ^{3}H could be used for identifying and quantifying adducts.

At about the same time that these initial experiments were completed, it became apparent from the work done on Task 2,that, as had previously been ascertained with rat keratinocyte cultures, cytotoxicity was readily observable in human cultures at exposure levels well below 50 uM BCES. Therefore, cultures of human keratinocytes, which had been grown in the presence of [^{3}H]GR, were exposed to 55 uM [^{14}C]BCES with the expectation that once adducts were identified, exposures at lower concentrations would be done.

Cells were exposed to 55 uM [14C]BCES for 30 min and the DNA was isolated by a procedure that utilized sodium dodecyl sulfate (SDS), sodium perchlorate and a "silica" technique (Nucleon II by Scotlab, Kirkshaws Road, Coatbridge, Strathclyde, Scotand ML5 8AD) instead of proteinase K and phenol for cell lysis and deproteinization.

The isolated DNA was heated at 70° C for 30 min at pH 3.5 and analyzed by gel permeation and HPLC. By gel filtration on the small column of BioRad P2 (for dimensions of the column, see the legend for Fig. 5), peaks of ultraviolet absorbance$_{254nm}$ were found in Fractions 5-7 and 9-11 and a shoulder in Fraction 7-9 (Fig. 15A). Unfortunately, although the BCES contained ^{14}C, the specific activity was low and the DNA was not adequately labeled with ^{14}C to be useful in identifying adducts. There were two peaks of ^{3}H (Fig. 15B). One peak was found in Fraction 5-8 with the maximum radioactivity in Fraction 7 and one, in Fraction 9-12 with the maximum in Fraction 10. The former peak had about 35% and the latter peak about 65% of the total radioactivity. Fractions 8, 9 and 10 from the BioRad P2 column were submitted, separately, to HPLC. As shown in Figure 16A, Fraction 8 showed a major peak of ultraviolet absorbance$_{254nm}$ at 14.6 min and a smaller peak at 11.9 min. On the other hand the major peak of ^{3}H was at 12.5 min and the lesser one at 15 min (Fig. 17A). (Note: As noted above, there is a delay of approximately 1 min from the time that a substance appears as absorbance in the flowing stream until it appears in collected fractions for assay of radioactivity.) Fraction 9 showed peaks at 9.6, 10.4, 11.3 and 14.2 min by absorbance (Fig.16B) but peaks at 10, 11.5 and 14.8 min by analysis of radioactivity (Fig. 17B). Fraction 10 had a major peak of absorbance at 9.4 min and a small peak at 10.5 min (Fig 16C) and radioactivity at 10 min (Fig. 17C). A standard sample of guanine eluted at 9.5 min by absorbance. The identity of the tritiated peak at 4 min shown in Figures 17A, B and C is unknown and is now considered to be irrelevant toxicologically (cf., discussion on Page 43). Also, the identities of the peaks of ^{3}H at 12.5 and 15 min in Figure 16A are unknown but must be derived from guanine since they were radioactive.

Table 3 compares the profiles of ^{3}H obtained when submerged cultures of human keratinocytes, whose DNA contained tritiated guanine residues, or isolated [^{3}H]DNA, with the label in guanine, were exposed to 300 uM BCES and then analyzed by gel permeation on BioRad P2. The table shows that 90% of the label was in the residual DNA while 6% was found in Fractions 24-31. About 0.2% was found at the position of the mono-adduct of guanine. Although most of the radioactivity in Fractions 24-31 appears to be toxicologically irrelevant some small portion probably resides in the di-guanyl-adduct.

Figure 15. Separation of ^{3}H and absorbance$_{254nm}$ by gel permeation of a hydrolysate of DNA isolated from cells previously labeled with [^{3}H]GR and exposed to 55 uM BCES. For technique of isolation of DNA, see the text. For separation technique, see the legend for Figure 5. Hydrolysis was carried out at pH 3.5 and 70° C for 30 min. A: absorbance; B: radioactivity (^{3}H).

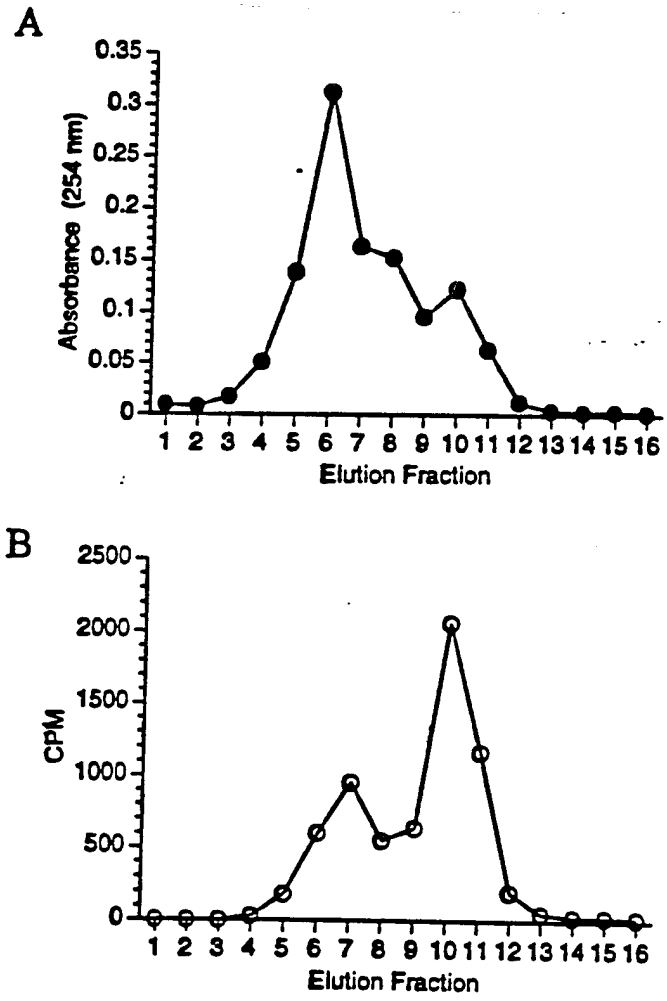
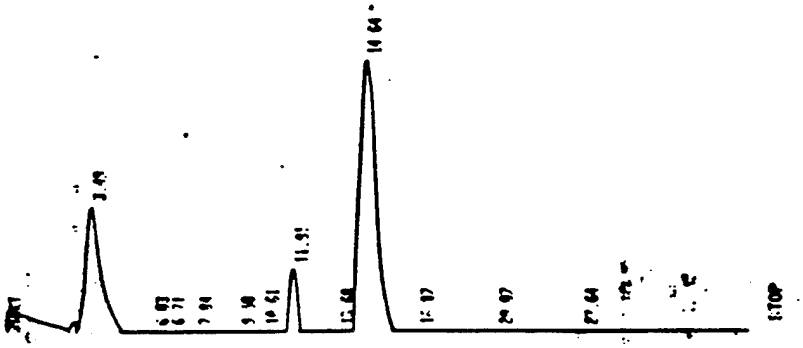
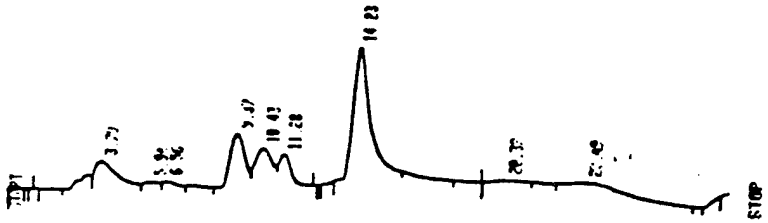


Figure 16. Profile of absorbance$_{254nm}$ from HPLC of Fractions 8, 9 and 10, respectively, obtained by gel permeation as shown in Figure 15. A: Fraction 8; B: Fraction 9; C: Fraction 10.

A



B



C

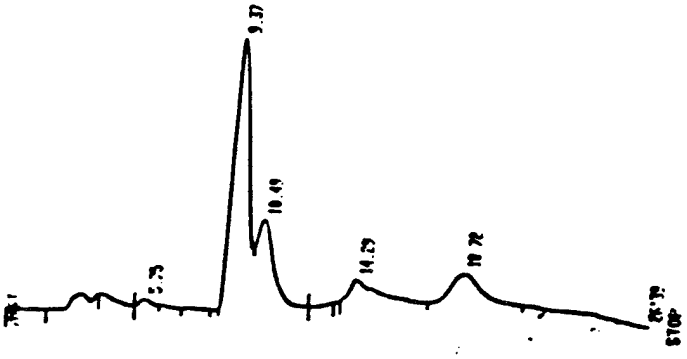


Figure 17. Profile of ^{3}H from HPLC of Fractions 8, 9 and 10, respectively, obtained from gel permeation as shown in Figure 14. A: Fraction 8; B: Fraction 9; C: Fraction 10.

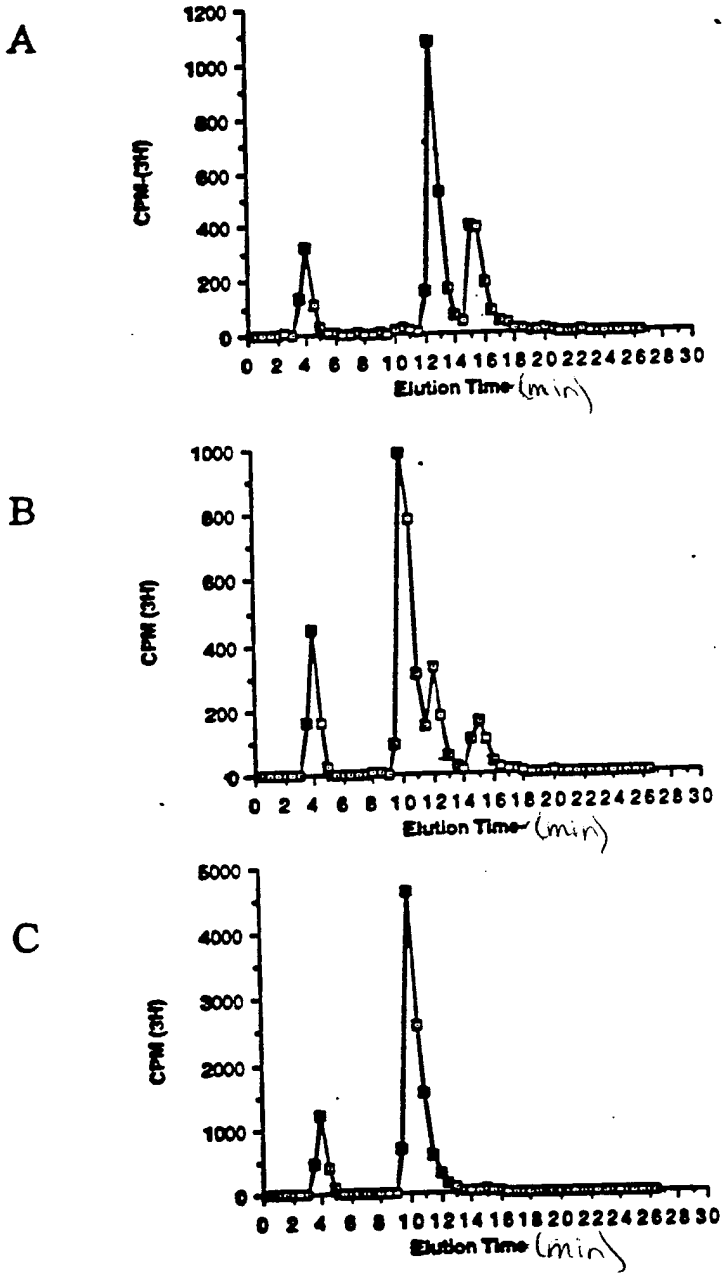


Table 3. Fractionation of cultured human keratinocyte DNA containing tritiated guanine residues, on a column of BioRad P2 after exposure to BCES followed by hydrolysis at pH 3.5 for 30 min at 70° C. For a description of the column and other technical details, see the legend for Figure 7.

Condition for Exposure	Exposure uM	Column Fractions 11-18	24-31	Eluted 38-41	Total Recovery#
		cpm*/fract.			
In vitro	300	23,200	6,150	156	30,400
		55,500	11,000	596	71.900
In the cell	300	69,000	4,500	159	76,400

#Includes the cpm in all fractions. *^{3}H

Conclusion: The data in Table 3 indicate that the same dose of BCES was less effective when applied to cells as when applied to isolated DNA. This was not a surprise since BCES alkylates protein and RNA as well as DNA. In general, the profiles of ^{3}H eluted from BioRad P2 were similar for the hydrolysate of DNA whether exposed in the isolated form or in the cell.

Task 4: To determine whether exposure to BCES decreases the efficacy of the repair system for BCES-mediated adducts in cells exposed to the mustard by

a) Transfecting monolayered cultures of human keratinocytes with SV40 viral DNA containing two mismatched bases and evaluating, against untreated controls, the effect of the exposure on the time-delay and rate of appearance of new viral DNA

In a previous study, Fan and Bernstein (1991) showed that, in African green monkey kidney (AGMK) cells, exposure to BCES caused a dose-responsive delay in the initiation of mismatched base repair. This parameter was measured by assaying the presence of new virus after cells were exposed and then transfected with SV40 DNA whose replication was temperature sensitive as a result of the presence of two mis-matched base pairs. The appearance of new viral DNA was monitored by the incorporation of [^{3}H]TdR into viral DNA

at the non-permissive temperature. Repair of at least one of the mis-matched base pairs was necessary to obtain stable large T antigen protein at the non-permissive temperature of 41° C. The presence of large T antigen is required for the replication of viral DNA. AGMK cells were used for transfection of SV40 DNA because efforts to use human keratinocytes had failed. It appeared that the failure was primarily a result of inadequate culture conditions for the keratinocytes rather than inadequate transfection.

The protocol for growing human keratinocytes submerged in medium containing a "low" or "normal" level of calcium ion was modified so that adequate cultures could be obtained consistently. The main change consisted of replacing the impure trypsin (Difco) previously employed with a purified trypsin (Sigma). Use of the purified trypsin for isolating cells from the tissue resulted in a larger percentage of viable basal cells than was true with the former enzymatic preparation. When the purified trypsin was used, there were fewer fibroblasts and differentiated cells in the isolated population. Centrifugation in a 38% Percoll density gradiant further purified the population. The altered protocol led to the consistent formation of a confluent monolayer in fewer days (i.e., 3-5 instead of 21 days) whichever level of calcium ion was used in the medium (i.e., whether production of a monolayer or a stratified culture was the objective). It then became possible to reinvestigate transfection of human keratinocytes with mutant SV40 viral DNA and, if successful, to use the system for evaluating the effect of exposure to BCES on the efficiency of mismatch-base repair in these human cells.

Samples of wild type and temperature-sensitive, mutant type SV40 viral DNA which were necessary for the proposed studies, were accumulated by growth in AGMK cells.

Because of the central role of large T antigen in the replication of the viral DNA, it was decided to investigate the use of an immuno-analytical assay for large T antigen rather than the incorporation of [^{3}H]TdR into viral protein as a measure of the cellular repair of the viral DNA probe. To develop the new assay as well as to determine the relative infection efficiency between AGMK cells and human keratinocytes, both cell types were infected using the procedure previously reported by Fan and Bernstein (1991). Ten million cells were plated in 60mm culture dishes and grown for 3 days at 37° C under 5% CO_2-air, to achieve confluence. SV40 wild type virus (2 X 10^9 PFU) was added for 90 min followed by 2 days of incubation. The cells were then harvested and 0.1 ml containing 3 X 10^6 cells was plated on glass cover slips. After 3 days of incubation, the cultures were washed with PBS, air-dried and dipped into a 3:7 mixture of methanol and acetone for 10 min at -20° C. After drying in air to remove the organic solvents, 20 ul of anti-T mouse serum were added to each cover slip for 30 min. Then after washing with PBS, 20 ul of rabbit anti-mouse IgG with attached fluorescein isothiocyanate (FITC) were added for 30 min. The cover slips were then washed, dried and viewed for fluorescence. Whereas 80% of the cells in the AGMK cultures showed fluorescence, the keratinocyte cultures showed no fluorescent cells.

The data clearly showed that the technology used for growing virus in the AGMK cells was not applicable to the keratinocytes.

Steinberg and Difendi (1979) had reported that human keratinocytes were more easily infected when they were in suspension rather than in monolayer culture. Therefore, infection of keratinocytes was attempted by adding SV40 to the initial suspension of cells before any cultivation. Otherwise, the procedure was the same as outlined above. Figure 18A shows the confluent layer of keratinocytes as viewed without the UV filter. Figure 18B shows the absence of fluorescence in uninfected cells. Figure 18C shows that 15-20% of the keratinocytes fluoresced under the conditions of infection used. Figure 18D shows the high percentage of AGMK cells that were infected while in monolayer.

Although infection of monolayer cultures of human keratinocytes was not successful, monolayer cultures were successfully transfected. DNA from wild type SV40 virus was used to transfect confluent cultures of keratinocytes grown for 3-5 days on glass coverslips without prior subcultivation. Serum free medium (Gibco) was used for the growth and Lipofectamine (Life Technologies) was used for the transfection according to the technique used by Fan and Bernstein (1991) for AGMK cells. Lipofection was allowed to proceed for 18 hr. The transfection medium was removed and the cultures were incubated further at 37° C for various times to allow the synthesis of the large T antigen. Table 4 indicates the number of fluorescent cells per microscope field observed at different times of incubation post-transfection. The appearance of fluorescence in the culture occurred essentially between 18 and 30 hr after transfection.

Conclusion: The technology necessary to pursue this task is now in hand. Monitoring the appearance of large T antigen by indirect immunofluorescence can be used to assay the repair of mismatched base pairs in viral DNA.

Transfection at 41° C with the mutant viral DNA (i.e., the DNA that has the two mismatched base pairs) is in progress. Nitrogen mustard will be used to complete the study if sulfur mustard is unavailable.

Task 5: To establish which adducts may be associated with cell death by

(a) Establishing a dose-responsive relationship between particular adducts and cell viability.

Cross-linked DNA is reported to be a source of lethality in cells exposed to cross-linking agents. Since the literature indicates that the di-guanyl-adduct is the

Figure 18. Immunofluorescence photomicrographs of the formation of large T antigen in cultures infected with wild type SV40 virus. A-C: cultures of human keratinocytes. D: Culture of AGMK cells infected with SV40 virus and viewed with the fluorescence filter. A: Submerged monolayer culture viewed without the fluorescence filter. B: Control culture viewed with the fluorescence filter. C: Infected culture viewed with the fluorescence filter. For technical details, see the text.

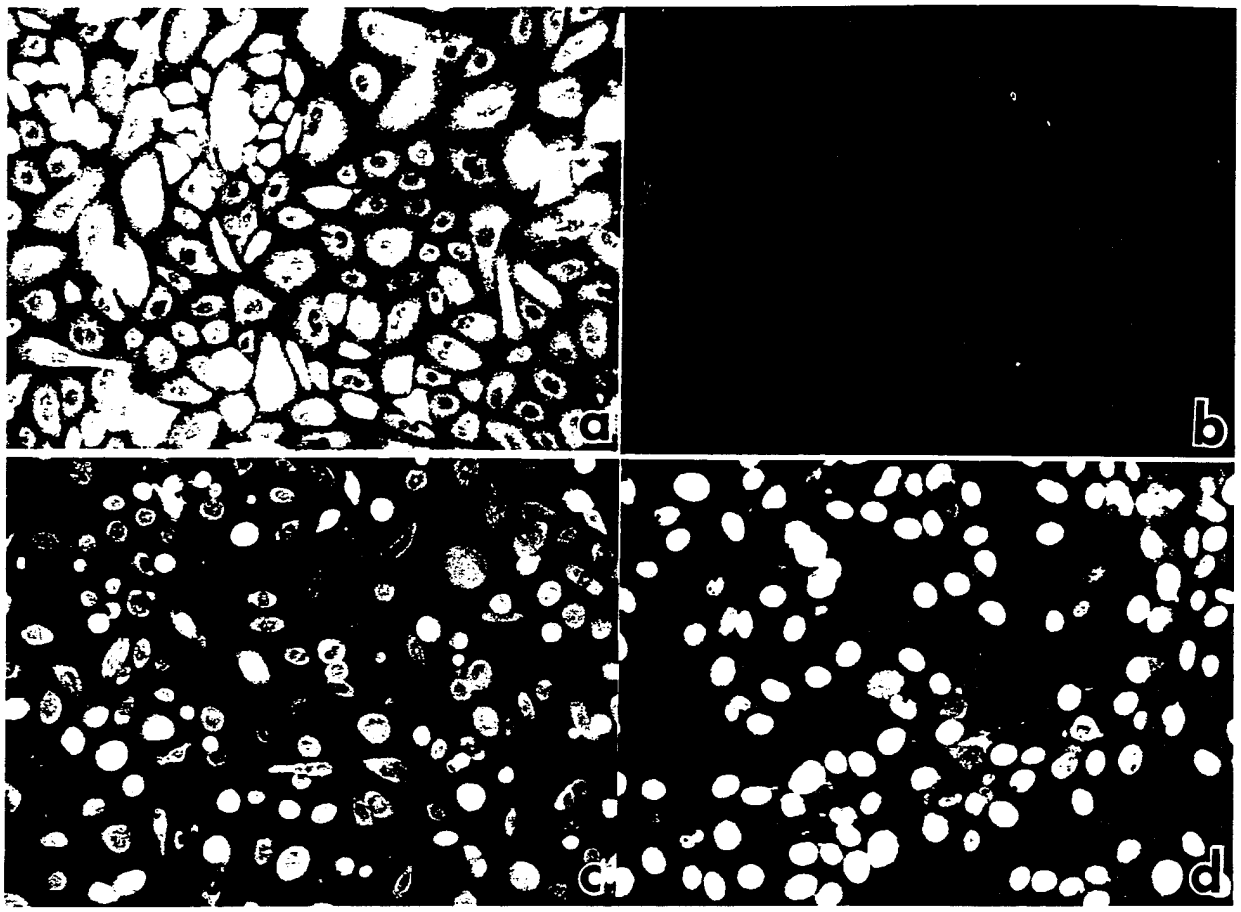


Table 4. Appearance of large T antigen in submerged cultures of human keratinocytes transfected with wild type SV40 viral DNA by lipofection. For details of procedure, see the text.

Hrs Post-transfection	Fluorescent cells per microscope-field
6	0
18	2±1
30	65±25
42	70±20
54	60±10
66	55±10

identity of one of the adducts involving guanine although the degree to which this adduct represents inter- or intra-strand cross-links is presently unknown, it was decided to determine the correlation, if any, between the appearance of cross-links, which represent inter-strand di-adducts in DNA, and loss of viability in submerged cultures of human keratinocytes exposed to different concentrations of BCES. In an effort to obtain a more convenient and, possibly, more valid marker for loss of viability than the technique involving exclusion of trypan blue, a study of the dose-responsive appearance of interleuken-1α (IL-1α) was also undertaken. For detai;ls of the technology used in these studies, see Section B, 7 and B, 8, above.

Table 5 shows the effect of BCES on viability in exposed cultures. At an exposure of 1 uM BCES, cell viability was normal after 24 hr but decreased to 74% by 72 hr post-exposure. At 10 uM, cell viability was decreased to 73% even at 2 hr post-exposure and continued to decrease through 72 hr. At the higher exposures, viability was even lower at 2 hr than after exposure to 10 uM BCES and decreased further through 72 hr.

Figure 19 shows the relationship between the concentration of BCES to which the cells were exposed and the percentage of cross-linked DNA determined immediately

Table 5. The percentage of viable cells in monolayer cultures of human keratinocyte cultures after exposure to BCES.

	Percentage of viable cells				
	uM BCES				
Hr Post-exposure	0	1	10	50	100
2	100	102	73	62	57
24	100	104	67	55	54
48	100	77	62	46	33
72	100	74	48	38	22

Eight million human epidermal basal cells were plated per well in 6-well plates. Low-calcium (0.08 mM) medium was used. After 4 days, cultures were exposed to 0-100 uM BCES at 37° C for 30 min. Cultures were then washed 2 times with PBS. One ml of medium was added and cultivation was resumed. Medium was changed every day. Cells were collected by trypsinization and counted using trypan blue. Data are the average of two separate experiments.

after exposure of human keratinocytes to the mustard. Exposure of cells to 1-10 uM BCES resulted in low and similar percentages of cross-linked DNA. Above 10 uM BCES, the DNA showed a linear dose-responsive increase in cross-linking.

Figure 20 shows a semi-log plot of the relationship between the quantity of IL-1α in cells and the percentage of viable cells in exposed cultures assayed at 72 hr post-exposure.

Figure 21 is a linear plot of the level of the cytokine at 72 hr after exposure against the percentage of cross-linking in the DNA immediately after exposure.

Figure 19. Formation of interstrand cross-links in DNA of submerged monolayer human keratinocyte cultures immediately after exposure to BCES. Data are the average of three samples. Cultures were washed with PBS and exposed to BCES in PBS for 30 min. After exposure cultures were washed with PBS and lysed in a lysing solution that was 400 mM Tris-HCl, 60 mM EDTA, 150 mM NaCl, 1% SDS, pH 8.0. DNA was isolated by the Nucleon silica procedure described in the text followed by alcohol precipitation. Interstrand cross-links were determined using ethidium bromide in the fluorescence assay described by Brent (1984).

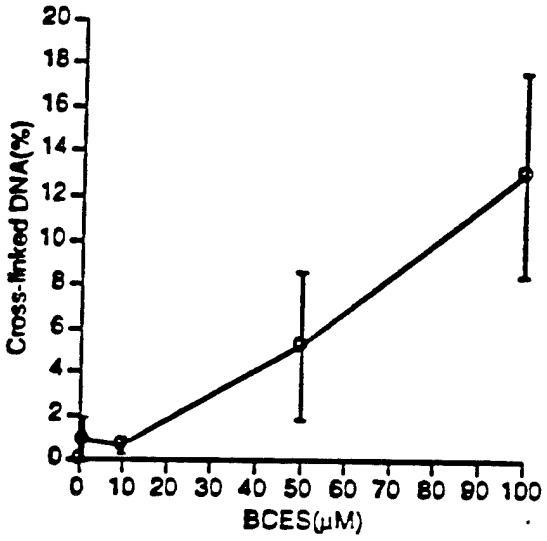


Figure 20. The correlation between the level of IL-1a in cells and cell vibility at 72 hr post-exposure to BCES. For additional technical details, see legends for Table 1 and Figure 19.

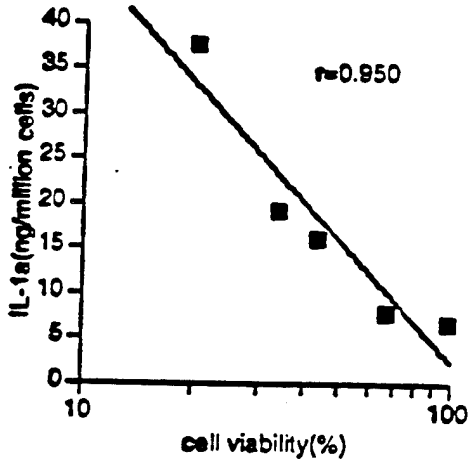
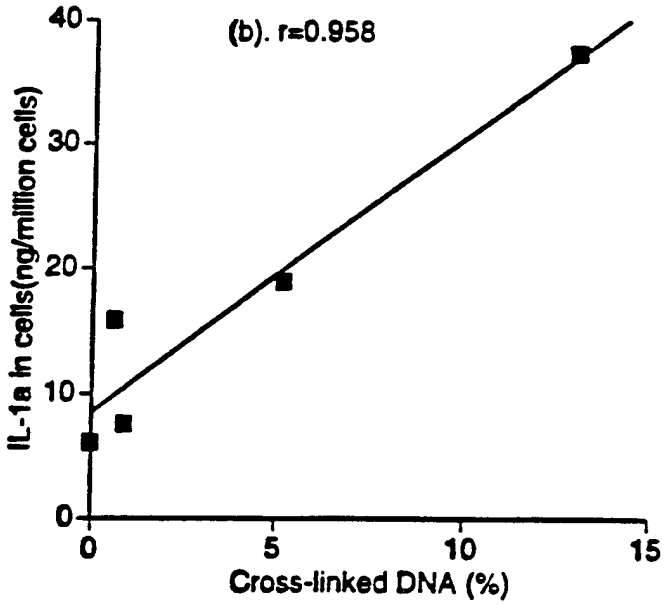


Figure 21. Relationship between cross-links in DNA determined immediately after exposure to BCES and the level of IL-1α in cells at 72 hr post-exposure. For technical details, see the legends to Table 1 and Figure 19.



Conclusion: The above data demonstrate that a correlative relationship exists between the immediate formation of cross-links (measured by the intercalation of ethidium bromide in quickly denatured DNA). the appearance of the cytokine at 72 hr post-exposure (measured by an ELISA assay for human IL-1α) and the percentage of viable cells - also determined at 72 hr post-exposure - (measured by loss of ability to exclude trypan blue). The level of cross-linking - and by extrapolation, inter-strand di-adducts - in the DNA that is imposed on the cell immediately upon exposure to BCES is a predictor of loss of viability in cells at 72 hr after that exposure. Furthermore, the level of IL-1a in the cell at 72 hr post-exposure can be used as a marker for the percentage of viable cells in a culture.

(b) **Establishing the nature of the repair process involved in recovery from low-level BCES-injury, e.g., base replacement, excision repair.**

Prior work in this laboratory by Ribeiro (Thesis, The University of Michigan, 1988) indicated that at relatively low levels of exposure to BCES, cells in monolayer cultures of rat keratinocytes incorporated radioactive deoxyguanosine (GdR) into their DNA whereas they did not do so with labeled thymidine (TdR). This observation was interpreted to indicate that replacement of damaged bases (e.g., guanine) rather than excision and replacement of large sections of the DNA chain represented the mode of repair after low-level exposure to BCES (see Section A, 2, d, 2), above, for a discussion of this matter).

A re-investigation of this hypothesis has been carried out using submerged multilayer cultures of primary human keratinocytes. In an initial experiment cells, grown for 3 days, were exposed for 30 min to 1 uM BCES and then were pulse-labeled with 1 uCi/ml of [^{3}H]GR or [^{3}H]TdR (added to growth medium) for 1 hr after different periods of incubation post-exposure. Labeling periods were 0-1, 2-3 and 4-5 hr. DNA was isolated and the contained ^{3}H per unit of A_{260nm} was determined. As shown in Figure 22, the incorporation of [^{3}H]GR into DNA was five times greater than [^{3}H]Tdr in the period 0-1 hr but was half as high in the periods 2-3 and 4-5 hr. In an effort to define the period of this initial high incorporation of GR, a similar experiment was carried out in which the pulse-labeling was to 2 uCi/ml for 30 min at 0-30,30-60, 60-90 and 90-120 min post-exposure. As shown in Figure 23, GR was incorporated into the DNA at a level that was 10 times greater than TdR in the period 0-30 min and slightly greater than two times higher in the next 30 min. Again the incorporation of GR was about half the level of TdR thereafter. Apparently, the high incorporation of [^{3}H]GR was an early event after exposure to BCES. Future studies will determine the dose- and time- (i.e., post-exposure) responsiveness of the incorporation of GR in relation to the incorporation of TdR.

Conclusion:These results support the hypothesis that after relatively low-levels of exposure to BCES, base replacement is the preferred mechanism for repair of DNA. The early incorporation of [^{3}H]GR appears to coincide with the early appearance of single-strand breaks in the DNA of cells exposed to 1 uM BCES (Ribeiro, et al., 1991) and both would be expected to occur according to the currently accepted mechanism for replacement of individual alkylated bases in DNA.

Figure 22. Pulse-labeling of DNA by [^{3}H]GR and [^{3}H]TdR in submerged cultures of human keratinocytes at different times post-exposure to 1.0 uM BCES. Pulse : 1 uCi/ml for 60 min after indicated point on graph. Exposure to BCES: 30 min. Percentage based upon incorporation of [^{3}H]GR at 0 time. Culture conditions: As noted for normal calcium medium in METHODS.

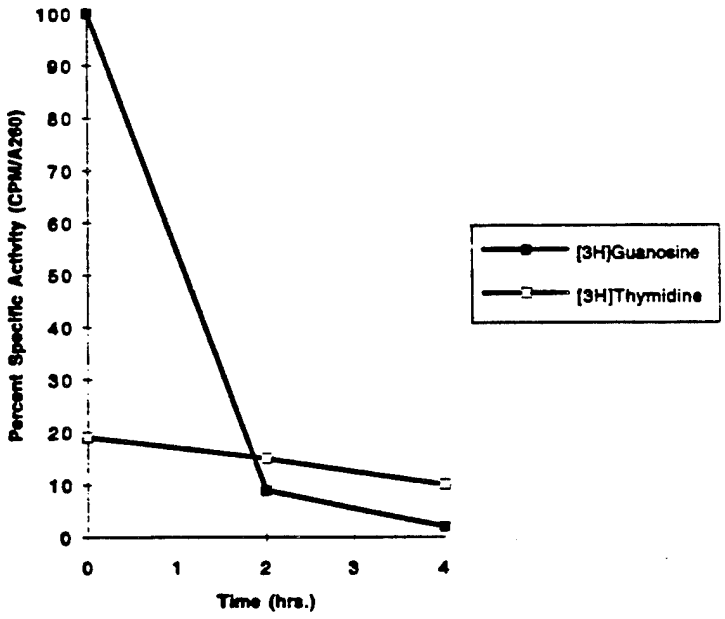
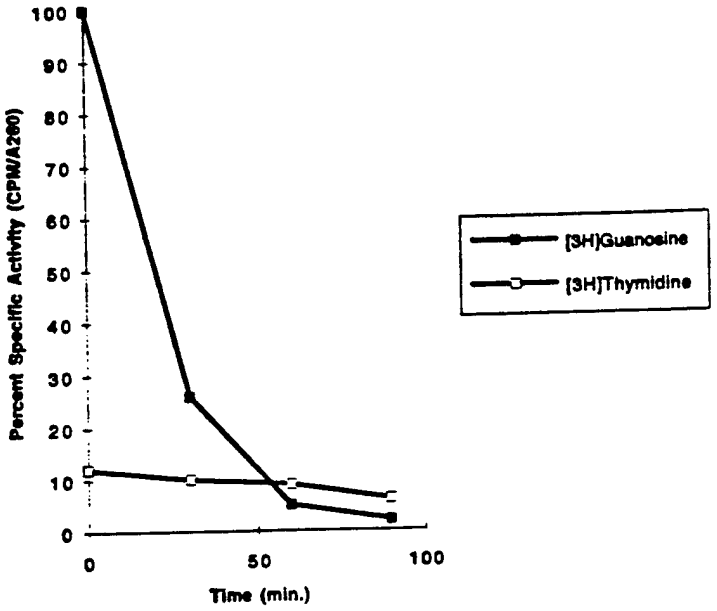


Figure 23 Pulse-labeling of DNA by [3H]GR and [3H]TdR in submerged cultures of human keratinocytes at different times post-exposure to 1.0 uM BCES. Pulse: 2 uCi/ml for 60 min after indicated point on graph. For other procedural details, see the legend to Figure 1.



Task 6: To evaluate the likelihood that the basal cell is more susceptible to damage from BCES than the differentiated cell because

a) The degree of alkylation of DNA is greater in the basal cell or the degree of repair is less

In work done previously with pseudo-epidermis derived from rat keratinocytes, Scavarelli-Karantsevalos found (cf., Thesis, The University of Michigan, 1989) that topical exposure to different levels of [^{14}C]BCES resulted in a larger amount of radioactivity per unit of DNA associated with the nucleic acid isolated from basal cells than was true for DNA isolated from differentiated cells. These data were interpreted to mean that the DNA was more susceptible to alkylation by BCES in basal than in differentiated cells. However, another possible explanation was that repair was faster in the differentiated than in the germinative cells.

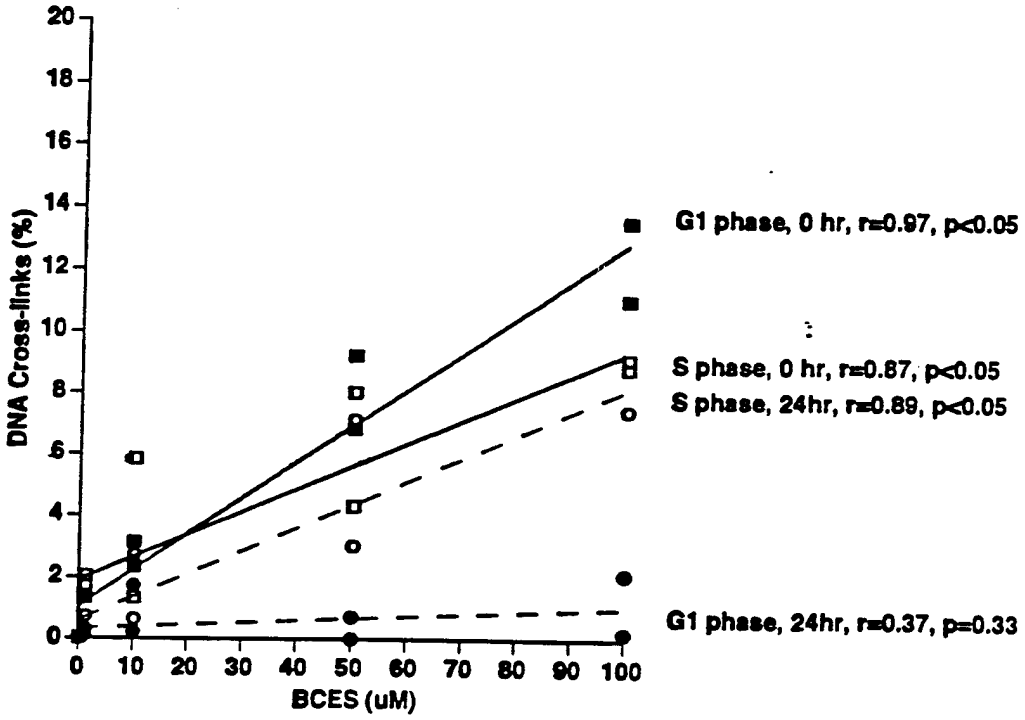
Using the nucleoid sedimentation technique (cf., Ribeiro, et al., Toxicol. Appl. Pharmacol., 111:342-351, 1991), Scavarellio-Karantsevalos (cf., Thesis, The University of Michigan, 1989) also quantified the presence of single-strand breaks in the DNA of basal cells and differentiated cells of stratified , cornified cultures exposed to BCES. (**Note:** Most of the single-strand breakage that appeared probably resulted from the DNA repair process i.e., the removal of alkylated bases from the DNA.) She showed that it took an exposure of 50 nmol/cm^2 to significantly introduce single-strand breaks in the DNA of the differentiated cells while only 10 nmol/cm^2 was necessary for the basal cells. These results suggested that the lower amount of radioactive BCES found with the DNA of the differentiated cells in the previous study was indeed a result of more effective repair of the damaged DNA in differentiated cells as compared with basal cells.During the period of this project,further evidence for this conclusion was sought.

To initiate a study of whether or not the DNA of the basal cell, where replication and transcription of DNA occurs, was more susceptible to alkylation by BCES than was the DNA of the differentiated cell, where only transcription occurs, A surrogate system was investigated in this regard. It seemed reasonable to assume that while undergoing replication, the DNA is more susceptible to alkylation than it is when being transcribed. To test this supposition, a study of the susceptibility of cellular DNA during the G1 and S phase of the cell cycle was evaluated in submnerged cultures of rat keeratinocytes. Flow cytometric experiments were carried out to see what effect BCES had on the DNA when cells were exposed to BCES while poised at the G1/S phase of the cell cycle, i.e., ready to undergo DNA replication, as compared with when cells were in the G1 phase, i.e., not set to undergo DNA replication but capable of DNA transcription. It was assumed that the DNA in some of the former cells would be in a similar condition as the DNA in the cells of the basal layer while the DNA of the latter cells would be in the same state as the DNA of differentiated cells.

Cells in submerged monolayer cultures were synchronized at the G1/S boundary of the cell cycle by incubation in the presence of Aph,The methodology for synchronization is described in Section B, 9, above. By the procedure used, 65% of the cells were collected at the G1/S interface. At that time 11% were in the G1 phase, 12% were in the S phase and 12% were in the G2+M phases. When Aph was removed, the cultures proceded through the cycle and by 10 hours after release from the block, 80% of the cells were in G1, 7% were in the S phase and 13% were in the G2+M phases. These determinations were made by flow cytometry as described in Section B, 10, above.

Since there was evidence from Task 5 that interstrand cross-links were related to loss of cell viability, interstrand cross-links were measured (as described in Section B, 9, above) in cells at the G1/S boundary and in G1 immediately after exposure to 0, 1, 10 or 50 uM BCES for 30 min. Figure 24 shows the dose-responsive increase in the percentage of

Figure 24. Relation between concentration of BCES and the percentage of cross-linked DNA found in submerged cultures of rat keratinocytes when cells were exposed to the mustard while they were at the G1/S boundary (S phase) or G1 phase of the cell cycle.



cross-linking in the DNA of the exposed cells at the two phases of the cell cycle. Contrary to expectation, the degree of interstrand cross-linking was not greater in G1/S cells. In fact, the cross-linking appeared to be slightly higher in differentiated cells. However, when the measurements were made 24 hr post-exposure, the degree of cross-linking had hardly changed in the G1/S phase cells while the level of cross-linking had decreased by over 90% in the G1 cells that had been exposed to 100 uM BCES. Given the assumptions that were made,these data supported the previous conclusion that differentiated cells were less susceptible to toxicity from BCES than were basal cells because of the more effective repair of the lesions in the DNA.

However,when this same technology was applied to topically exposed human pseudo-epidermis, as shown by the data in Table 6, similar results were not found. Pseudo-epidermis was exposed by topical application of 25 or 100 nmol of BCES/cm^2 for 30 min. Either immediately post-exposure or after 24 hr of incubation, the basal and differentiated cell populations were separated and their DNA isolated as described in Section B, above. Interstrand cross-linking was then determined in the DNA from the two cell types. Whereas the cross-linking was similar in the two cell types immediately after exposure, the best repair

Table 6. The effect of topically applied BCES on the levels of interstrand cross-links in the DNA of basal and differentiated cells in human pseudo-epidermis.

BCES ($nmol/cm^2$)	Cell Type	Hr Post-exposure	%Cross-linked DNA
25	Basal	0	1.1
		24	0.0
	Differentiated	0	1.6
		24	1.4
100	Basal	0	2.3
		24	0.0
	Differentiated	0	1.9
		24	2.9

Cultures were exposed for 30 min at 35° C by topical application of BCES in PBS. Cultures were then washed and analyzed or incubated for 24 hr in MEM at the air-liquid interface. Interstrand cross-links were determined by measuring the residual fluorescence from intercalated ethidium bromide in the DNA after heat-denaturation and rapid cooling to destroy hydrogen-bonded double-strandedness of the DNA. Ethidium bromide intercalates and fluoresces in double-stranded DNA that is formed rapidly under the denaturing conditions used because of the presence of interstrand cross-links.

appeared to have occurred in the basal rather than the differentiated cells. The same levels of cross-linking were seen in the differentiated cells at both times while the cross-linking decreased to the non-detectable level at 24 hr in the DNA from basal cells.

Conclusion: There seems to be no question that the cross-linking imposed on the DNA of differentiated and basal cells was similar when human pseudo-epidermis was topically exposed to BCES. This leaves "better repair" as the logical explanation for why the DNA of differentiated cells had a lower level of radioactivity than did the DNA of basal cells when the pseudo-epidermis was exposed to labeled mustard. However, the results of an experiment done to test this conclusion did not support this explanation. However, the finding of no cross-linking in the DNA from basal cells 24 hr post-exposure may have been an artifact. It is possible that the most traumatized basal cells did not survive the isolation procedure and, therefore, did not contribute their DNA to the pool of DNA that was analyzed. Certainly, as shown in Figure 4, there is obvious destruction of the basal layer by 24 hr post-exposure of pseudo-epidermis to 100 uM BCES.

(b) The levels of NAD^+ and NADH are drastically decreased by BCES in the germinative cell but not in the differentiated cell.

Papirmeister, et al. (1985) formulated a mechanism for vesication in skin exposed topically to BCES that included, successively, (a) alkylation of DNA; (b) enzymatic insertion of single-strand breaks in the DNA as a result of DNA repair; (c) activation of poly(ADP-ribose) polymerase; (d) depletion of NAD^+ which is a substrate for this polymerase; (e) disturbances in normal carbohydrate metabolism, increased proteolysis, and, ultimately, (f) cellular death. In support of this hypothesis, exposure to BCES lowered the level of NAD^+ in human skin grafted to athymic mice (Gross, et al, 1985) and niacinamide, an inhibitor of poly(ADP-ribose) polymerase, partially protected cultured keratinocytes against this depletion of NAD^+ and against cytotoxicity for a short time (Smith, et al., 1990).

This facet of the current project was designed to test the role, if any, of depletion of total NAD (i.e., NAD^+ + NADH) in the destruction of basal cells in stratified, cornified cultures exposed topically to BCES at a level of 50 nmol/cm^2 - the level at which total destruction of the basal cell layer in cultures of cells derived from rat skin is seen. However, initially this aspect of the project has been carried out in submerged cultures of keratinocytes. The cultures were grown in a medium containing about 2 mM Ca^{2+}. Under these conditions, early stratification occurred but cornification was not achieved.

For these experiments, quantification of DNA was done with the "DABA" (i.e., 3,5-diaminobenzoic acid dihydrochloride) assay (Setaro and Morley, 1976)). Total NAD was determined as described in Section B, 6, above.

The experiments were first tried with cultures of human keratinocytes. However, as can be seen from the data in Table 7, the levels of total NAD found in different untreated cultures varied widely depending on the donor and the degrees of confluence and stratification of the cultures. Since the variation was too great to allow a credible evaluation of the effects of exposure to BCES, attention was turned to cultures of keratinocytes derived from the skin of newborn rats.

Keratinocytes from the skin of newborn rats were isolated, purified and plated as described by Ku and Bernstein (1988). Basal cells, obtained from rat skin by trypsinization and Percoll density gradient sedimentation, were suspended at a population of 1.6-1.8 X 10^6 cells/ml of medium and plated at 0.5 ml/well in plastic 16 mm 4-well dishes. Medium was changed at 24 hr after plating and then every 48 hr.

Figures 25 A, B, and C present data on the total NAD/well, the ug DNA/well and the pmol NAD/ug DNA in rat cultures grown submerged in about 2 mM Ca^{2+} over a period of 9 days. The data in Figures 25 A and B were the basis for the calculated values shown in Figure 25 C.

The figure shows that with the exception of the level for the first day (i.e., 24 hr after plating), the total NAD content of the culture normalized per unit of DNA varied between about 30 and 65 pmol/ug DNA. It is probable that early in the period , e.g., at 2-3 days, the cultures were primarily proliferating monolayers as is indicated by the increase in the ug DNA/well at 1-3 days (Figure 25 B).

Thereafter, the cultures were stratified and the major fraction of cells was probably differentiated. Note the relatively constant level of DNA/well after 4 days. Actually, the total NAD/well varied only between 500 and 675 pmol/well during the entire period of the experiment (Figure 25 A). After these data were obtained, it was decided to use this system for studies of the effect of BCES on total NAD/ug DNA.

Cells were isolated, purified and plated as noted above. On the second day after plating, the medium was decanted and replaced with BCES in PBS for 30 min at 37°C. Thereafter, the cultures were washed with PBS, further cultivated for 24, 48 or 144 hr submerged in medium containing 2.0 mM Ca^{2+} and prepared for assay. Control preparations were exposed to PBS alone.

Table 8 presents the data obtained on the effect of 10, 50 or 100 uM BCES on the total NAD and the NAD/ug DNA in the cultures. In 2-day old cultures, no decrease in the ug DNA/dish was seen at 24 hr after exposure to 50 uM BCES. A significant decrease in the amount of DNA/well was first seen only 48 hr after exposure to this level of BCES. In both cases, however, there was no significant decrement in the pmol NAD/ug DNA. It required an exposure to 100 uM BCES to cause a decrease in the NAD level in 2-day old cultures. This decrease was seen at 24 hr after exposure and was enhanced 48 hr after treatment. Although a significant decrease in the level of DNA/well occurred by 144 hr, no significant change was noted in the NAD level at this time after exposure to 50 uM BCES.

Table 7. Total NAD levels in submerged, stratified and monolayer cultures of human keratinocytes. Determinations of total NAD were made on stratified and monolayer cultures derived from the same donor. Total NAD is shown in pmol/ug DNA to control for possible differences in cell number among the various cultures. For procedural details, see the text.

Donor No.	% Confluence	Total NAD (pmol/ug DNA)	
		Monolayer Culture	Stratified Culture
10	100	200	-
14	"	131	118
15	"	142	-
19	"	554	203
10	70-90	97	-
14	"	122	142
17	"	242	169
18	"	382	349
17	50	103	173
18	"	353	468

Although the available data need further confirmation, a comparison of the data for 2-day old and 6-day old cultures appears to show that the older culture, in which a large fraction of the population is differentiated, is less sensitive to BCES than is the 2-day old culture, in which most of the cells are germinatively competent.

Earlier work has shown that mitotic activity is not reinitiated in submerged monolayer cultures (i.e., cultures grown for 24 hr in medium with 0.1 mM Ca^{2+}) exposed to as little as 5 uM BCES between 1-6 days after exposure and the cultures ultimately die. However, in 2-day old cultures (grown in medium with 2 mM Ca^{+}),

Figure 25. Content of total NAD and DNA in submerged cultures of keratinocytes derived from the skin of the newborn rat over the first 9 days of growth in medium containing 2.0 mM Ca^{2+}. A - pmol total NAD/well; B - ug DNA/well, and C - pmol total NAD/ug DNA calculated from the data in A and B. For procedural details, see the text.

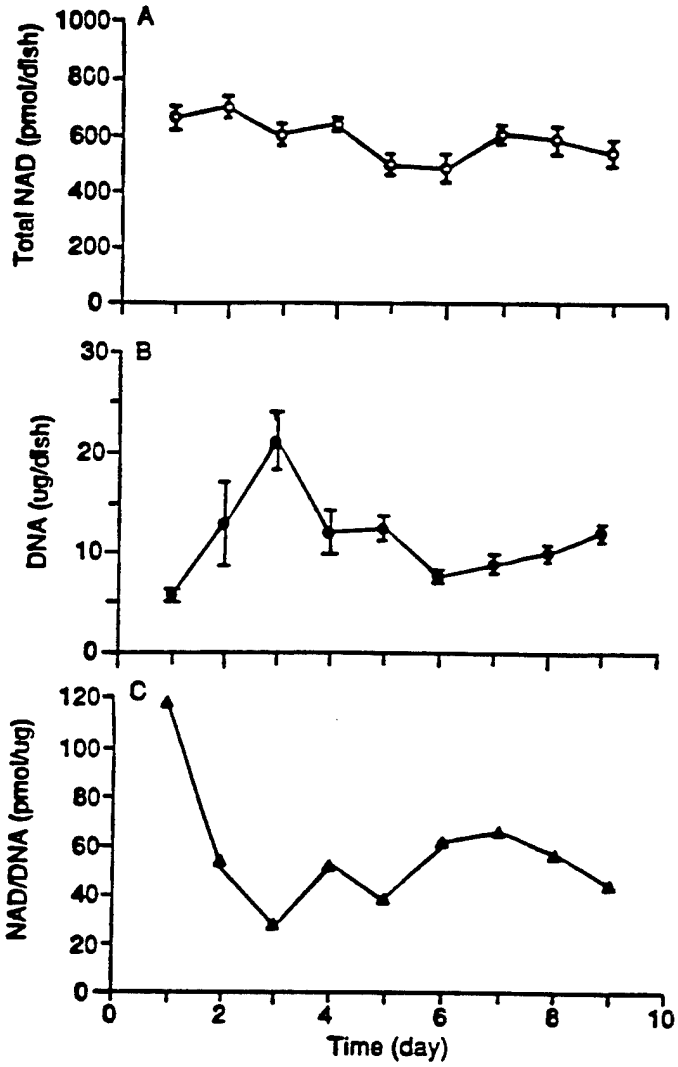


Table 8. Effect of BCES on total NAD in submerged cultures of rat keratinocytes exposed to BCES and incubated for 24 or 48 hr in medium with 2 mM Ca2PT.

Days after Plating for Exposure	Hr after Exposure for Analysis Analysis	Treatment	Total DNA (ug/22 mm dish)	Total NAD/DNA (pmol/ug)
2	24	Control	33±7	28±4
		100 uM BCES	31±3	22±7**
	48	Control	32±5	32±4
		100 uM BCES	20±3***	9±2***
6	24	Control	25±2	38±5
		100 uM BCES	21±4***	30±4**
	48	Control	25±2	41±3
		100 uM BCES	14±5***	34±7*
2	24	Control	33±6	30±3
		50 uM BCES	32±5	25±5
	48	Control	32±4	30±
		50 uM BCES	26±3***	28±4
6	24	Control	27±1	37±3
		50 uM BCES	27±3	28±2***
	48	Control	26±2	39±6
		50 uM BCES	20±3***	40±4
2	48	Control	35±6	28±4
		50 uM BCES	28±4***	27±4
	144	Control	27±2	36±3
		50 uM BCES	20±1***	39±3
2	48	Control	34±6	29±5
		10 uM BCES	32±2**	31±4
	144	Control	25±1	36±4
		10 uM BCES	20±2***	39±2

Cells were exposed to PBS alone (control) or BCES in PBS at 37°C for 30 min on the second or sixth day after plating. For other procedural details, see the text. S.D. =p<0.01(*); =p< 0.005 (**); =p< 0.001 (***).

no significant decrease in the pmol of NAD/ug DNA was observed after an exposure to 50 uM BCES whether analyzed 24, 48, or 144 hr post-exposure to the mustard. This was true even though the culture underwent a significant loss of cells as represented by a decrease in the amount of DNA/culture by 48 hr post-exposure. An exposure to 100 uM BCES was necessary to get an effect on the level of pmol NAD/ug DNA in this type of culture. On the other hand, exposure of a 6-day old culture to 50 uM BCES did lead to a significant decrease in pmol of total NAD/ug DNA at 24 hr post- exposure which, however, disappeared by 48 hr post-exposure. Therefore, the data in Table 3 do not seem to support the hypothesis that a dramatic loss of total NAD/cell preceeds the cytotoxicity of BCES in proliferating keratinocytes in submerged culture. This conclusion is supported by the data shown in Figure 17. This figure shows that exposure of a 2-day old submerged culture (grown in medium containing 2 mM Ca^{2+}) to 50 uM BCES resulted in a gradual loss of cells from the culture (based on the total DNA in the culture). By 48 hr after exposure to the mustard, the total DNA in the treated cultures had decreased by 25% relative to the control cultures. During the same period the level of NAD had increased. The value for pmol total NAD/ug DNA was 15% greater in the treated cultures than in the control cultures. However, the conclusion that loss of total NAD does not cause cytotoxicity would be further strengthened by actually demonstrating a cessation of DNA replication, inhibition of mitosis and cell death after an exposure of 50 uM BCES.

Conclusion: Exposure of 2-day-old submerged cultures of rat keratinocytes to 50 uM BCES resulted in a 20-25% loss of DNA from the culture without a concomitant decrease in the level of NAD/cell at 48 hr post-exposure. This loss of DNA represented a loss of cells from the culture. Therefore, at least in the case of the culture, no evidence was obtained to support the hypothesis that a drastic decrease in the level of NAD/cell preceeds destruction of cells exposed to BCES as has been proposed by Papirmeister, et al. (1985).

Task 7: To determine whether the molecular lesion in the germinative cells that disrupts the normal balance between epidermal cellular proliferation and differentiation is metabolic or informational in nature by:

(α) Obtaining a preparation of active 2D6 monoclonal antibody against the 55 kD keratin found in normal basal cells.

Prior work in this laboratory had shown that submerged monolayered cultures of rat keratinocytes exposed to 10 uM BCES produced a keratin of abnormal Mr which bound to a monoclonal antibody, 2D6, which had been raised against a normal keratin (Locey, Thesis, The University of Michigan, 1988). Task 7 was designed to confirm these observations and ascertain whether or not they resulted from abnormal information in the DNA caused by exposure to BCES.

Unfortunately, the amount of 2D6 available in the laboratory was small and the hybridoma clone for this monoclonal antibody had not survived several years of storage in liquid nitrogen. Efforts to develop a new hybridoma that produced a monoclonal

with the same specificity as 2D6 were unsuccessful. However, a monoclonal antibody, 7B4, was obtained which had a different specificity than 2D6 but appeared to be even more useful than 2D6 for the purpose of this task.

7B4 was shown by Western blotting to bind to an approximately 55 kD protein which was isolated from monolayer cultures of keratinocytes that had been exposed to 10 uM BCES 24 hr earlier (Fig. 26; Lane 8). The protein was extracted with Tris buffer containing SDS and was not seen in extracts from unexposed cells (Fig. 26; Lane 7). No 7B4-reactive protein was isolated from the basal cells of the rat epidermis (Fig. 26; Lane 3). The same result was obtained from basal cells derived from rat pseudo-epidermis (i.e., a stratified, cornified culture of keratinocytes) grown on a Puropore membrane at the air-liquid interface (Fig. 26; Lane 6). The protein was found in extracts of the entire pseudo-epidermis or from the differentiated cell population (Fig. 26: Lanes 4 and 5) but was not present in similar preparations made from the epidermis (Fig. 26; Lanes 1 and 2).

When used to stain frozen sections of rat skin, 7B4 decorated the cornified cell layer (Fig. 27A). The antibody was localized in the tissue section by using a second antibody (i.e. goat anti-mouse antibody) labeled with a fluorescent probe. The antibody decorated the pseudo-epidermis in a similar location as was seen with the epidermis (Fig. 27B). As shown in Figure 28, 7B4 did not bind to cells in unexposed monolayer cultures of keratinocytes but did bind to nearly all cells in monolayer cultures exposed to BCES.

Since 7B4 bound only to cells in the upper layers of the tissue and not cells in the basal or spinous layers of an unexposed culture, the binding in an exposed culture clearly suggested that a major commitment of the culture to differentiation had occurred as a result of exposure to BCES.

The finding of the protein in preparations of differentiated cells derived from the pseudo-epidermis although staining of cross-sections of pseudo-epidermis did not show staining below the cornified layers is confusing. Perhaps the preparation of differentiated cells was contaminated with cornified cells.

Figure 26. Evaluation of the binding of 7B4 monoclonal antibody to keratins extracted from (E) the epidermis of the newborn rat, (PE) pseudo-epidermis from rat keratinocytes, i.e., the stratified, cornified keratinocyte culture grown at the air-liquid interface, and (M) monolayer cultures of Keratinocytes grown submerged in a medium low in calcium ion. Keratinocytes, isolated from the respective tissue or culture were separated by SDS-PAGE on 7% polyacrylamide gels then transferred to nitrocellulose membranes for staining with 7B4 antibody. A second antibody with attached peroxidase was used to monitor the presence of 7B4. Basal and differentiated cells were separated by centrifugation in a self-forming gradient from 38% Percoll. c: control; e: 24 hr after exposure to 10 uM BCES for 30 min; w: whole tissue; d: differentiated cells; b: basal cells. For other details see the text.

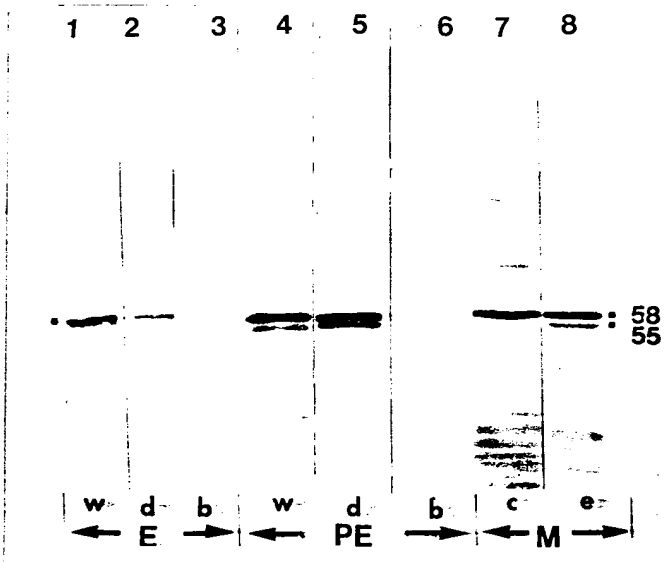


Figure 27: Cryostat cross-sections of the skin (A, B) of the newborn rat and pseudo-epidermis (C, D) grown from rat keratinocytes on Puropore microporous membranes. The cultures were grown submerged for 7 days and at the air-liquid interface for 14 days. In both cases, similar sections were stained with hematoxylin and eosin (A, C) and 7B4 antibody followed by a second antibody with a fluorescent ligand (B, D). x560. cc: cornified layer; b: basal layer. Space between cornified and basal layers in the immunofluorescence photomicrograph of pseudo-epidermis is a result of handling sections during the 5-hr staining procedure.

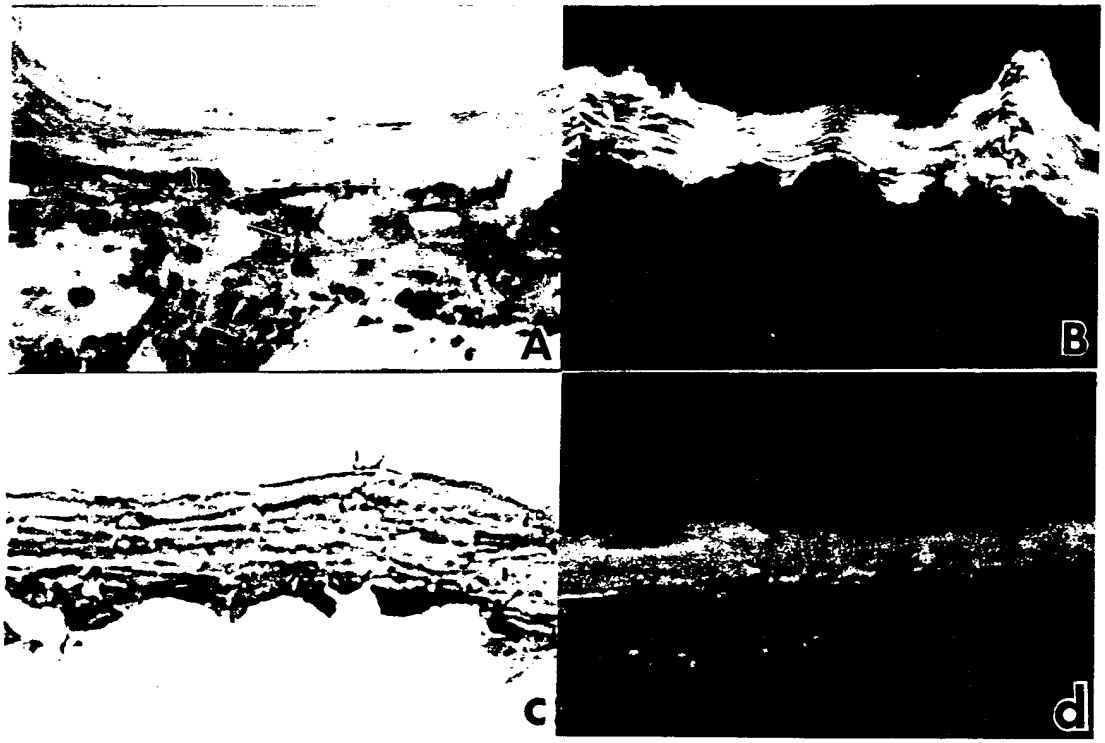
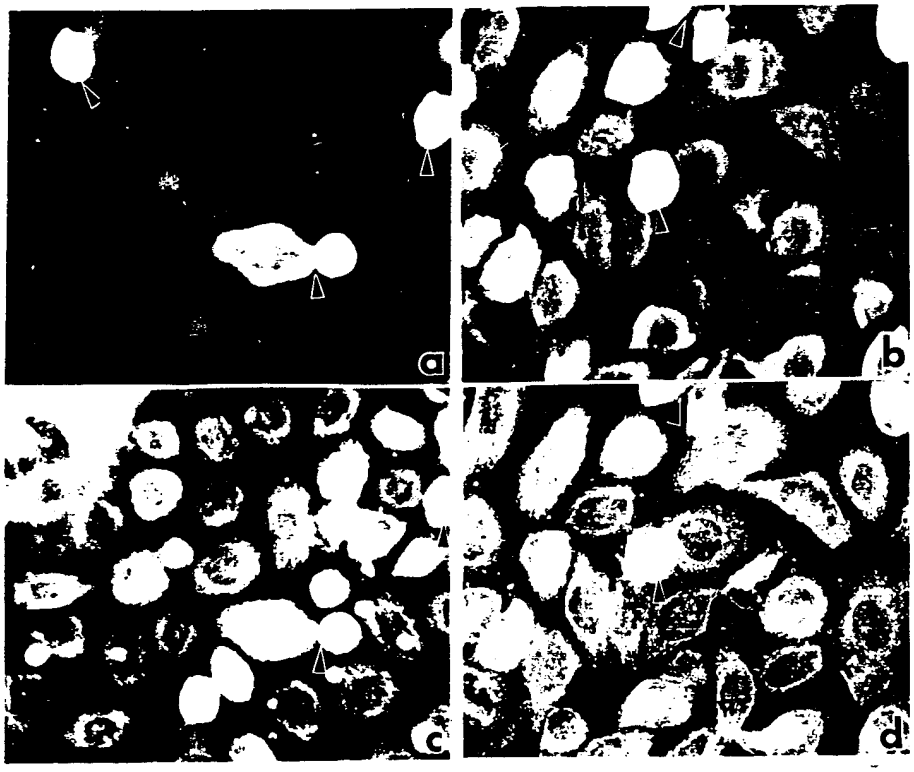


Figure 28. 7B4-stained rat keratinocytes after 2 days of cultivation in low Ca2+ MEM. a,b - cells visualized by indirect immunofluorescence. c,d - cells visualized without the fluorescence filter. a,c - control culture. b,d - after exposure to 10 uM BCES. Note the non-specific staining of cells marked by arrows.



D. General conclusions and recommendation for further study:

Vesication in human skin exposed to BCES includes the loss of the germinative population in the basal layer of the epidermis as a major and early component of the pathogenic process. The process appears to become irrevocable within a few minutes after the exposure but the pathology becomes evident only after a delay.

The mission of this project has been to answer the following questions which are associated with the cutaneous toxicology of BCES:

i) Why does the process become irreversible within a few minutes after the exposure?

ii) Why does the basal layer degenerate in a time- and dose-dependent manner?

iii) Why do the differentiated layers remain relatively unaffected by levels of topically applied BCES that result in the destruction of of the basal layer?

Answers to these questions have been sought by focusing on the primary interaction between the toxicant and the cell, i.e., the imposition of damage to DNA resulting from alkylation of the nucleic acid by BCES; the cell's ability to repair the damage; the physiological result of the failure to adequately repair the DNA, the influence of dosage and time post-exposure in the development of the lesion in DNA, and the possible difference in the susceptibility to this influence exhibited by the DNA of basal and differentiated cells. Pseudo-epidermis and submerged cultures of human and rat keratinocytes have been used in this project to eliminate any secondary systemic effects or effects from other cell types in the skin. Although convenient for the purposes of this investigation, use of these *in vitro* systems require that any conclusions drawn from this project be considered in the light of the artificiality involved.

Based upon inhibition of the incorporation of [^{3}H]TdR into DNA, a measure of DNA synthesis, in human pseudo-epidermis exposed to very low levels of BCES, data derived from Task 2 allow the interpretation that DNA is an early, if not the first, target for BCES in the cutaneous epidermis. The cessation of DNA synthesis seems to be a general response of the cell to damage to its DNA. The most prevalent adducts in the DNA of keratinocytes resulting from alkylation by BCES appear to involve guanine as has been reported generally for DNA in various species and types of cells (Task 3). After exposure to low levels of BCES, a "base replacement" mechanism of repair seems to have preference. The results reported for Task 5 support this conclusion. The high rate of incorporation of [^{3}H]GR, relative to the incorporation of [^{3}H]TdR, in submerged cultures of human keratinocytes immediately after exposure to 1 uM BCES, can be interpreted to signal the replacement of single damaged bases rather than the excision of a sequence of bases including the damaged one. If this supposition is correct, at higher doses of BCES, the two tracers should be

incorporated in the repair mode at similar rates. This experiment has not been done as yet. Also, yet to be determined is the dose of mustard which causes the mode of repair to change from base replacement to excision repair.

Intuitively, one would expect that replacement of a single damaged base would result in repair with higher fidelity than would the replacement of a sequence of bases. Correspondingly, repair done by excising a sequence of bases could result in the insertion of informational errors with consequent alteration of normal physiological events. Supporting this view is the finding in Task 7 that exposure of a submerged monolayer of rat keratinocytes to 10 uM BCES results in precocious differentiation within the culture thus decreasing the germinative population of cells. Figure 28 shows that a monolayer culture, consisting almost entirely of germinatively-capable cells, did not bind the 7B4 monoclonal antibody which in epidermis and pseudo-epidermis decorated the cornified layer (Fig. 27). However, two days after exposure to 10 uM BCES, nearly all the cells in the monolayer bound this antibody. In fact, as a result of the exposure to BCES, a specific keratin was present which was not found in unexposed cultures and which was normally present only in differentiated cells of the pseudo-epidermis (Fig. 26). It seems reasonable to consider that pathogenesis becomes irreversable at a level of BCES which triggers repair by excision repair and a consequental insertion of informational ahanges.

Although other explanations may be offered, these observations can be interpreted to indicate that as a result of exposure to BCES, germinative cells became committed to differentiation, a type of programmed cell death, because of changes in the informational content of their DNA. Thus two phenomena may play a role in the BCES-mediated loss of germinative cells. At a relatively lower level of BCES, the germinative population may be decreased by the early commitment of cells to differentiation, a process which obviates their ability to divide. At higher levels of exposure, this decrease in the population of germinatively-capable cells may be enhanced by the generalized destruction, i.e., necrosis, of these cells. Dose-response studies of these two phenomena could contribute to the credibility of this hypothesis but have not been done to date. If disruption of the regulatory role of DNA in the mitotic process were indeed the major target of BCES, the greater stability of differentiated cells as compared with basal cells would be more understandable. Interstrand cross-links in DNA could prevent replication of the nucleic acid for cell division in the basal layer but might not prevent the differentiated cell from transcribing its DNA for metabolic activity. A dose-response study of the effect of BCES on the biosynthesis of protein in relation to its effect on the biosynthesis of DNA was done in Task 2. The data in Tables 1 and 2 indicate that the latter biosynthetic process was more drastically affected at relatively low topical exposures of pseudo-epidermis to BCES than was the formation of protein. Yet, as was shown in Task 6, interstrand cross-links were inserted into the DNA of both cell types to a similar extent at a somewhat higher level of exposure to the mustard.

As a result of the work done on this project, it seems reasonable to conclude that the data generated are consistent, although they do not prove, the hypothesis which was formulated for this inquiry. Alkylation of DNA by BCES initiates the pathogenic response in the epidermal germinative population. The severity of the effect depends on the

ability of the cell to repair its damaged DNA with high fidelity and this ability is compromised in a dose-responsive manner. The decrease in germinatively competent cells occurs because of a commitment to differentiation as well as necrosis depending on the dose of BCES involved.

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F. Publications During the Reporting Period:

The following papers reporting results from studies on this project by this laboratory were accepted for publication or have been already published during the project period:

Fan, L., and Bernstein, I. A. (1991) Effect of bis-(beta-Chloro-ethyl)sulfide (BCES) on Base Mismatch Repair of DNA in Monkey Kidney Cells. Toxicol. Appl. Pharmacol., 111, 233-241.

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